THE PROKARYOTES

Third Edition

A Handbook on the Biology of Bacteria:

Bacteria: Firmicutes, Cyanobacteria

Edited by

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Volume 4

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A Handbook on the Biology of Bacteria

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Volume 4: Bacteria: Firmicutes, Cyanobacteria

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Springer

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URLs in *The Prokaryotes*: Uncommon Web sites have been listed in the text. However, the following Web sites have been referred to numerous times and have been suppressed for aesthetic purposes: www.bergeys.org; www.tigr.org; dx.doi.org; www.fp.mcs.anl.gov; www.ncbi.nlm.nih.gov; www.genome.ad.jp; www.cme.msu.edu; umbbd.ahc.umn.edu; www.dmsz.de; and www.arb-home.de. The entirety of all these Web links have been maintained in the electronic version.

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Preface

Each of the first two editions of *The Prokaryotes* took a bold step. The first edition, published in 1981, set out to be an encyclopedic, synoptic account of the world of the prokaryotes—a collection of monographic descriptions of the genera of bacteria. The Archaea had not yet been formalized as a group. For the second edition in 1992, the editors made the decision to organize the chapters on the basis of the molecular phylogeny championed by Carl Woese, which increasingly provided a rational, evolutionary basis for the taxonomy of the prokaryotes. In addition, the archaea had by then been recognized as a phylogenetically separate and distinguishable group of the prokaryotes. The two volumes of the first edition had by then expanded to four. The third edition was arguably the boldest step of all. We decided that the material would only be presented electronically. The advantages were obvious and persuasive. There would be essentially unlimited space. There would be no restrictions on the use of color illustrations. Film and animated descriptions could be made available. The text would be hyperlinked to external sources. Publication of chapters would be seriati—the edition would no longer have to delay publication until the last tardy author had submitted his or her chapter. Updates and modifications could be made continuously. And, most attractively, a library could place its subscribed copy on its server and make it available easily and cheaply to all in its community. One hundred and seventy chapters have thus far been presented in 16 releases over a six-year period. The virtues and advantages of the online edition have been borne out. But we failed to predict the affection that many have for holding a bound, print version of a book in their hands. Thus, this print version of the third edition shall accompany the online version.

We are now four years into the 21st century. Indulge us then while we comment on the challenges, problems and opportunities for microbiology that confront us.

Moselio Schaechter has referred to the present era of microbiology as its third golden age—the era of "integrative microbiology." Essentially all microbiologists now speak a common language. So that the boundaries that previously separated subdisciplines from each other have faded: physiology has become indistinguishable from pathogenesis; ecologists and molecular geneticists speak to each other; biochemistry is spoken by all; and—mirabile dictu!—molecular biologists are collaborating with taxonomists.

But before these molecular dissections of complex processes can be effective there must be a clear view of the organism being studied. And it is our goal that these chapters in *The Prokaryotes* provide that opportunity.

There is also yet a larger issue. Microbiology is now confronted with the need to understand increasingly complex processes. And the modus operandi that has served us so successfully for 150 years—that of the pure culture studied under standard laboratory conditions—is inadequate. We are now challenged to solve problems of multimembered populations interacting with each other and with their environment under constantly variable conditions. Carl Woese has pointed out a useful and important distinction between empirical, methodological reductionism and fundamentalist reductionism. The former has served us well; the latter stands in the way of our further understanding of complex, interacting systems. But no matter what kind of synoptic systems analysis emerges as our way of understanding host–parasite relations, ecology, or multicellular behavior, the understanding of the organism as such is sine qua non. And in that context, we are pleased to present to you the third edition of *The Prokaryotes*.

Martin Dworkin
Editor-in-Chief

Foreword

The purpose of this brief foreword is unchanged from the first edition; it is simply to make you, the reader, hungry for the scientific feast that follows. These four volumes on the prokaryotes offer an expanded scientific menu that displays the biochemical depth and remarkable physiological and morphological diversity of prokaryote life. The size of the volumes might initially discourage the unprepared mind from being attracted to the study of prokaryote life, for this landmark assemblage thoroughly documents the wealth of present knowledge. But in confronting the reader with the state of the art, the Handbook also defines where more work needs to be done on well-studied bacteria as well as on unusual or poorly studied organisms.

This edition of *The Prokaryotes* recognizes the almost unbelievable impact that the work of Carl Woese has had in defining a phylogenetic basis for the microbial world. The concept that the ribosome is a highly conserved structure in all cells and that its nucleic acid components may serve as a convenient reference point for relating all living things is now generally accepted. At last, the phylogeny of prokaryotes has a scientific basis, and this is the first serious attempt to present a comprehensive treatise on prokaryotes along recently defined phylogenetic lines. Although evidence is incomplete for many microbial groups, these volumes make a statement that clearly illuminates the path to follow.

There are basically two ways of doing research with microbes. A classical approach is first to define the phenomenon to be studied and then to select the organism accordingly. Another way is to choose a specific organism and go where it leads. The pursuit of an unusual microbe brings out the latent hunter in all of us. The intellectual challenges of the chase frequently test our ingenuity to the limit. Sometimes the quarry repeatedly escapes, but the final capture is indeed a wonderful experience. For many of us, these simple rewards are sufficiently gratifying so that we have chosen to spend our scientific lives studying these unusual creatures. In these endeavors many of the strategies and tools as well as much of the philosophy may be traced to the Delft School, passed on to us by our teachers, Martinus Beijerinck, A. J. Kluyver, and C. B. van Niel, and in turn passed on by us to our students.

In this school, the principles of the selective, enrichment culture technique have been developed and diversified; they have been a major force in designing and applying new principles for the capture and isolation of microbes from nature. For me, the "organism approach" has provided rewarding adventures. The organism continually challenges and literally drags the investigator into new areas where unfamiliar tools may be needed. I believe that organism-oriented research is an important alternative to problem-oriented research, for new concepts of the future very likely lie in a study of the breadth of microbial life. The physiology, biochemistry, and ecology of the microbe remain the most powerful attractions. Studies based on classical methods as well as modern genetic techniques will result in new insights and concepts.

To some readers, this edition of the *The Prokaryotes* may indicate that the field is now mature, that from here on it is a matter of filling in details. I suspect that this is not the case. Perhaps we have assumed prematurely that we fully understand microbial life. Van Niel pointed out to his students that—after a lifetime of study—it was a very humbling experience to view in the microscope a sample of microbes from nature and recognize only a few. Recent evidence suggests that microbes have been evolving for nearly 4 billion years. Most certainly those microbes now domesticated and kept in captivity in culture collections represent only a minor portion of the species that have evolved in this time span. Sometimes we must remind ourselves that evolution is actively taking place at the present moment. That the eukaryote cell evolved as a chimera of certain prokaryote parts is a generally accepted concept today. Higher as well as lower eukaryotes evolved in contact with prokaryotes, and evidence surrounds us of the complex interactions between eukaryotes and

prokaryotes as well as among prokaryotes. We have so far only scratched the surface of these biochemical interrelationships. Perhaps the legume nodule is a pertinent example of nature caught in the act of evolving the "nitrosome," a unique nitrogen-fixing organelle. Study of prokaryotes is proceeding at such a fast pace that major advances are occurring yearly. The increase of this edition to four volumes documents the exciting pace of discoveries.

To prepare a treatise such as *The Prokaryotes* requires dedicated editors and authors; the task has been enormous. I predict that the scientific community of microbiologists will again show its appreciation through use of these volumes—such that the pages will become "dog-eared" and worn as students seek basic information for the hunt. These volumes belong in the laboratory, not in the library. I believe that a most effective way to introduce students to microbiology is for them to isolate microbes from nature, i.e., from their habitats in soil, water, clinical specimens, or plants. *The Prokaryotes* enormously simplifies this process and should encourage the construction of courses that contain a wide spectrum of diverse topics. For the student as well as the advanced investigator these volumes should generate excitement.

Happy hunting!

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CHAPTER 1.2.1

The Genera *Staphylococcus* and *Macrococcus*

FRIEDRICH GÖTZ, TAMMY BANNERMAN AND KARL-HEINZ SCHLEIFER

Introduction

The name *Staphylococcus* (staphyle, bunch of grapes) was introduced by Ogston (1883) for the group micrococci causing inflammation and suppuration. He was the first to differentiate two kinds of pyogenic cocci: one arranged in groups or masses was called "*Staphylococcus*" and another arranged in chains was named "Billroth's *Streptococcus*." A formal description of the genus *Staphylococcus* was provided by Rosenbach (1884). He divided the genus into the two species *Staphylococcus aureus* and *S. albus*. Zopf (1885) placed the mass-forming staphylococci and tetrad-forming micrococci in the genus *Micrococcus*. In 1886, the genus *Staphylococcus* was separated from *Micrococcus* by Flügge (1886). He differentiated the two genera mainly on the basis of their action on gelatin and on relation to their hosts. Staphylococci liquefied gelatin and were parasitic or pathogenic or both whereas micrococci were variable in their action on gelatin and were saprophytic. The genera *Staphylococcus*, *Micrococcus* and *Planococcus*, containing Gram-positive, catalase-positive cocci, were later placed in the family Micrococcaceae. Evans et al. (1955) proposed separating staphylococci from micrococci on the basis of their relation to oxygen. The facultative anaerobic cocci were placed in the genus *Staphylococcus* and the obligate aerobic cocci in the genus *Micrococcus*. By the mid-1960s, a clear distinction could be made between staphylococci and micrococci on the basis of their DNA base composition (Silvestri and Hill, 1965). Members of the genus *Staphylococcus* have a DNA G+C content of 33–40 mol%, whereas members of the genus *Micrococcus* have a high G+C content of around 70 mol%. Further studies have shown that staphylococci can be distinguished from micrococci and other catalase-positive cocci on the basis of their cell wall composition (Schleifer and Kandler, 1972; Endl et al., 1983), cytochrome profile (Faller et al., 1980) and menaquinone pattern (Collins and Jones, 1981), susceptibility to lysostaphin and erythromycin (Schleifer and Kloos, 1975b), bacitracin (Falk and Guering, 1983), and fura-

zolidone (Baker, 1984). Comparative immunochemical studies of catalases (Schleifer, 1986), DNA-DNA hybridization studies, DNA-rRNA hybridization studies (Schleifer et al., 1979; Kilpper et al., 1980), and comparative oligonucleotide cataloguing of 16S rRNA (Ludwig et al., 1981) clearly demonstrated the epigenetic and genetic difference of staphylococci and micrococci. Members of the genus *Staphylococcus* form a coherent and well-defined group of related species that is widely divergent from those of the genus *Micrococcus*. Until the early 1970s, the genus *Staphylococcus* consisted of three species: the coagulase-positive species *S. aureus* and the coagulase-negative species *S. epidermidis* and *S. saprophyticus*, but a deeper look into the chemotaxonomic and genotypic properties of staphylococci led to the description of many new staphylococcal species. Currently, 36 species and several subspecies are recognized in the genus *Staphylococcus* (Table 1).

The genus *Macrococcus* has been described on the basis of comparative 16S rRNA analysis, DNA-DNA hybridization studies, ribotype patterns, cell wall composition, and phenotypic characteristics (Kloos et al., 1998a). Macrococci can be separated from staphylococci on the basis of a generally higher DNA G+C content (38–45 mol%), absence of cell wall teichoic acids (with the possible exception of *M. caseolyticus*), unique ribotype patterns, and generally larger cells. Members of the genus *Macrococcus* are also oxidase positive whereas most staphylococci (exceptions: *S. lentus*, *S. sciuri* and *S. vitulus*) are oxidase negative. There are four species in the genus *Macrococcus* (Table 1).

Isolation Techniques

Isolation of *S. aureus* from Foods

Staphylococcus aureus has been confirmed to be the causative agent of many cases of severe food poisoning; therefore, its presence in foods is of major concern. *Staphylococcus aureus* is very susceptible to heat treatment and most sanitiz-

Table 1. List of species and subspecies in the genera *Macrococcus* and *Staphylococcus*.

Species name	References[a]
Macrococcus bovicus	Kloos et al., 1998a
M. carouselicus	Kloos et al., 1998a
M. caseolyticus	Schleifer et al., 1982
	Kloos et al., 1998a
M. equipercicus[T]	Kloos et al., 1998a
Staphylococcus arlettae	Schleifer et al., 1984
S. *auricularis*	Kloos and Schleifer, 1983a
S. *aureus*[T]	Rosenbach, 1884
S. *aureus* subsp. *anaerobius*	De la Fuente et al., 1985
S. *aureus* subsp. *aureus*	De la Fuente et al., 1985
S. *capitis*	Kloos and Schleifer, 1975b
S. *capitis* subsp. *capitis*	Bannerman and Kloos, 1991
S. *capitis* subsp. *urealyticus*	Bannerman and Kloos, 1991
S. *caprae*	Devriese et al., 1983
S. *carnosus*	Schleifer and Fischer, 1982
S. *carnosus* subsp. *carnosus*	Probst et al., 1998
S. *carnosus* subsp. *utilis*	Probst et al., 1998
S. *chromogenes*	Devriese et al., 1978
S. *cohnii*	Schleifer and Kloos, 1975c
S. *cohnii* subsp. *cohnii*	Kloos and Wolfshohl, 1983b
S. *cohnii* subsp. *urealyticus*	Kloos and Wolfshohl, 1983b
S. *condimenti*	Probst et al., 1998
S. *delphini*	Varaldo et al., 1988
S. *epidermidis*	Winslow and Winslow, 1908
S. *equorum*	Schleifer et al., 1984
S. *felis*	Igimi et al., 1989
S. *fleurettii*	Vernozy-Rozand et al., 2000
S. *gallinarum*	Devriese et al., 1983
S. *haemolyticus*	Schleifer and Kloos, 1975c
S. *hominis*	Kloos and Schleifer, 1975b
S. *hominis* subsp. *hominis*	Kloos et al., 1998b
S. *hominis* subsp. *novobiosepticus*	Kloos et al., 1998b
S. *hyicus*	Devriese et al., 1978
S. *intermedius*	Hájek, 1976a
S. *kloosii*	Schleifer et al., 1984
S. *lugdunensis*	Freney et al., 1988
S. *lutrae*	Foster et al., 1997
S. *muscae*	Hájek et al., 1992
S. *pasteuri*	Chesneau et al., 1993
S. *piscifermentans*	Tanasupawat et al., 1992
S. *pulverei* (= *S. vitulinus*)	Petras, 1998
	Zakrzewska-Czerwinska et al., 1995
S. *saccharolyticus*	Kilpper-Bälz and Schleifer, 1981
S. *saprophyticus*	Schleifer and Kloos, 1975c
S. *saprophyticus* subsp. *bovis*	Hájek et al., 1996
S. *saprophyticus* subsp. *saprophyticus*	Hájek et al., 1996
S. *schleiferi*	Freney et al., 1988
S. *schleiferi* subsp. *coagulans*	Igimi et al., 1990
S. *schleiferi subsp. schleiferi*	Igimi et al., 1990
S. *sciuri*	Kloos et al., 1976a
S. *sciuri* subsp. *carnaticus*	Kloos et al., 1997
S. *sciuri* subsp. *lentus*	Kloos et al., 1997
S. *sciuri* subsp. *rodentium*	Kloos et al., 1997
S. *sciuri* subsp. *sciuri*	Kloos et al., 1997
S. *simulans*	Kloos and Schleifer, 1975b
S. *succinus*	Lambert et al., 1998
S. *vitulinus*	Webster et al., 1994
S. *warneri*	Kloos and Schleifer, 1975b
S. *xylosus*	Schleifer and Kloos, 1975c

Abbreviation: [T], type species.
[a]First description.

ing agents. Hence, when it or its enterotoxins are found in processed foods, poor sanitation is usually indicated. Detailed procedures for preparing food samples for analysis, isolating and enumerating *S. aureus*, and detecting staphylococcal enterotoxins in foods can be found in the following texts: *Compendium of Methods for the Microbiological Examination of Foods* (Downes and Ito, 2001), *Official Methods of Analysis of AOAC International* (Horowitz, 2000), and *Bacteriological Analytical Manual* (BAM; United States Food and Drug Administration, 1995). In addition to the AOAC (Association of Official Analytical Chemists) approved microslide test method (Horowitz, 2000), it is possible to detect enterotoxins directly in culture and in contaminated foods by the following rapid methods: radioimmunoassay (RIA) (Miller et al., 1978), enzyme-linked immunosorbent assay (ELISA), and reverse passive latex agglutination (RPLA). An ELISA kit, available from Tecra Diagnostics (Roseville, Australia), is distributed by International Bioproducts, Inc. (Redmond, WA, USA), and an RPLA is available from Oxoid (Columbia, MD, USA). Molecular methods are being investigated for their usefulness in detecting staphylococcal enterotoxin. For example, Western immunoblotting has been used to detect staphylococcal enterotoxin A (Rasooly and Rasooly, 1998). In this procedure, the staphylococcal enterotoxin A (native or heat-denatured) is separated by size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a membrane and then the membrane is probed with antibodies. The polymerase chain reaction (PCR) is an additional molecular method being studied for its ability to detect *S. aureus* and staphylococcal enterotoxins from food samples (McLauchlin et al., 2000; Atanassova et al., 2001; Tamarapu et al., 2001). One potential problem for PCR is the possibility of false-negative reactions due to PCR inhibitors that might be present in some foods.

Several conventional procedures for isolating *S. aureus* from foods are described below.

NONSELECTIVE ENRICHMENT PROCEDURES It is often necessary to use nonselective enrichment procedures for the detection of *S. aureus* in processed foods, especially when it is suspected that the food contains a small number of cells that may have been injured, e.g., as a result of heating, freezing, desiccation, or storage, and whose growth could be inhibited by toxic components of a selective enrichment media. The following nonselective (repair) enrichment procedure is appropriate for this use:

Nonselective Enrichment of *S. aureus* (Downes and Ito, 2001).

Transfer a 50-ml aliquot of a 1:10 dilution of the food sample homogenate to 50 ml of double-strength trypticase soy broth (TSB). Incubate the preparation for 3 h at 35–37°C. Then add 100 ml of a single-strength TSB containing 20% NaCl. Incubate for 24 ± 2 h at 35–37°C. Transfer 0.1-ml aliquots of the culture to each of duplicate Baird-Parker agar plates, and spread the inoculum, so as to obtain isolated colonies. Incubate the inoculated plates for 46 ± 2 h at 35–37°C. Select two or more colonies suspected to be *S. aureus* from each plate. *Staphylococcus aureus* colonies are usually 1.5 mm in diameter, jet-black to dark gray, smooth, convex, have entire margins and off-white edges, and may show an opaque zone and/or a clear halo extending beyond the opaque zone. Test selected colonies for coagulase activity. Results should be reported as *S. aureus* present or absent in 5 g of food, following the results of coagulase testing or clumping factor testing.

Coagulase Test (Horowitz, 2000; Downes and Ito, 2001).

Transfer colonies to tubes containing 0.2 ml of brain heart infusion (BHI) broth. Incubate culture suspensions 18–24 h at 35–37°C. Add 0.5 ml of reconstituted coagulase plasma with ethylenediaminetetraacetic acid (EDTA) and mix thoroughly. (The plasma is reconstituted according to manufacturer's directions. If not available, desiccated coagulase plasma (rabbit) is reconstituted and disodium dihydrate EDTA is added to final concentration of 0.1% in reconstituted plasma.) Incubate at 35–37°C and examine periodically during a 6-h interval for clot formation. Any degree of clot formation is considered a positive reaction. Small or poorly organized clots may be observed by gently tipping tube so that liquid portion of reaction mixture approaches lip of tube; clots will protrude above liquid surface. Coagulase-positive cultures are considered to be *S. aureus*. Test positive and negative controls simultaneously with cultures of unknown coagulase reactivity. Recheck doubtful coagulase test results on BHI cultures which have been incubated at 35–37°C for > 18 but ≤ 48 h.

This procedure is recommended by the AOAC for the identification of *S. aureus* isolated from foods. With this procedure, false-positive tests may occur with mixed cultures, but this will probably be avoided if only well-isolated colonies typical of *S. aureus* are chosen. On rare occasions, coagulase-negative mutants of *S. aureus* may be present in foods and overlooked by the above procedures, and the presence of other coagulase-positive staphylococci, such as *S. intermedius* and certain strains of *S. hyicus*, may be misrepresented as *S. aureus* using the coagulase test alone.

Baird-Parker agar base when supplemented with egg yolk tellurite enrichment is recommended for the detection and enumeration of coagulase-positive staphylococci in foods.

Baird-Parker Agar

Basal medium:

Tryptone	10.0 g
Beef extract	5.0 g
Yeast extract	1.0 g
Glycine	12.0 g
Lithium chloride · 6H$_2$O	5.0 g
Agar	20.0 g

This basal medium may be special-ordered from Difco Laboratories, Detroit, MI. Suspend ingredients in 950 ml distilled water. Boil to dissolve completely. Dispense 95.0 ml portions in screw-capped bottles. Autoclave 15 min at 121°C. Adjust final pH to 6.8–7.2 at 25°C.

Egg Yolk Tellurite Enrichment

Soak eggs in aqueous mercuric chloride 1:1000 for not less than one min. Rinse in sterile water and dry with a sterile cloth. Aseptically crack eggs and separate whites and yolks. Blend yolk and sterile physiological saline solution (3 + 7 v/v) in high-speed sterile blender for 5 s. Mix 50.0 ml of blended egg yolk with 10.0 ml of filter-sterilized 1% potassium tellurite. Mix and store at 2–8°C. Bacto egg-tellurite enrichment is a commercial preparation available from Difco Laboratories.

Preparation of Plates

Add 5.0 ml of prewarmed (45–50°C) enrichment to 95 ml of melted basal medium, which has been adjusted to 45–50°C. Mix well (avoiding bubbles), and pour 15.0–18.0 ml into sterile 15 × 100 mm Petri dishes. Plates can be stored at 2–8°C in plastic bags for 4 weeks. Immediately prior to use, spread 0.5 ml per plate of 20% solution of (membrane) filter-sterilized sodium pyruvate and dry plates at 50°C for 2 h or 4 h at 35°C with agar surface uppermost.

SELECTIVE ENRICHMENT PROCEDURES Selective enrichment is recommended for raw food ingredients and unprocessed foods expected to contain < 100 *S. aureus* cells/g and a large population of competing species. The recommended procedure of the AOAC is widely accepted and uses the most probable number technique.

Most Probable Number (MPN) Technique (Horowitz, 2000).

Inoculate three tubes of trypticase soy broth with 10% NaCl and 1% sodium pyruvate at each test dilution with 1-ml aliquots of decimal dilutions of sample. Highest dilution of sample must give a negative endpoint. Incubate 48 h ± 2 h at 35°C. Using 3-mm loop, transfer 1 loopful from each tube showing growth to dried Baird-Parker medium. Vortex-mix tubes before streaking if growth is visible only on bottom or sides of tubes. Streak inoculum to obtain isolated colonies. Incubate 48 h at 35–37°C. For each plate showing growth, transfer 1 colony suspected to be *S. aureus* to BHI broth. With sterile needle, transfer

colonies to tubes containing 0.2 ml of brain heart infusion (BHI) broth and to agar slants containing any suitable maintenance medium, e.g., trypticase soy agar, standard plate-count agar, etc. Incubate BHI culture suspensions and slants 18–24 h at 35°C. The BHI culture suspensions are used as inocula for the coagulase test (described above), and the slant cultures are used for ancillary tests or repeats of the coagulase test, if results are questionable. Report *S. aureus*/g as MPN/g, according to tables of MPN values (United States Food and Drug Administration, 1995).

Direct Surface Plating Procedures These procedures are sometimes preferred over the MPN technique for the detection of *S. aureus* in raw or unprocessed foods as they are more rapid and are regarded by some investigators to be more accurate than MPN.

Surface Plating Procedure for the Enumeration of S. aureus *(Horowitz, 2000)* For each dilution to be plated, aseptically transfer 1 ml of sample suspension to triplicate plates of Baird-Parker agar and distribute the 1 ml of inoculum equally over the triplicate plates (e.g., 0.4, 0.3, and 0.3 ml). Aseptically, spread the inoculum over the surface of the agar. Avoid the extreme edges of the plate. Maintain the plates in an upright position until the inoculum is adsorbed by the medium (about 10 min on properly dried plates). If the inoculum is not readily adsorbed, plates may be placed in an incubator in an upright position for about 1 h before inverting. Invert plates and incubate 45–48 h at 35–37°C. Select plates containing 20–200 colonies unless plates at only lower dilutions (> 200 colonies) have colonies with the typical appearance of *S. aureus*. If several types of colonies appear to be *S. aureus*, count the number of colonies of each type and record counts separately. When plates at the lowest dilution plated contain < 20 colonies, they may be used. If plates containing > 200 colonies have colonies with the typical appearance of *S. aureus* and typical colonies do not appear on plates at higher dilutions, use these plates for enumeration of *S. aureus*, but do not count nontypical colonies. Select one or more colonies of each type counted and test for coagulase production. Coagulase-positive cultures may be considered to be *S. aureus*. Add the number of colonies on triplicate plates represented by colonies giving a positive coagulase test, and multiply the total by the same dilution factor. Report this number as *S. aureus* per gram of product tested. The sensitivity of this procedure may be increased by using larger volumes (> 1 ml) distributed over > 3 replicate plates. Plating of two

or more decimal dilutions may be required to obtain plates with the desired number of colonies per plate.

Direct enumeration of coagulase-positive *S. aureus* can be made on Baird-Parker agar containing rabbit plasma-fibrinogen tellurite (Boothby et al., 1979) or Baird-Parker agar without egg yolk to which a tempered pork plasma-fibrinogen overlay agar has been added (Hauschild et al., 1979). In place of Baird-Parker agar, some laboratories have reported the successful use of tellurite polymyxin egg yolk agar (Crisley et al., 1964), Kaliumrhodanid (i.e., potassium thiocyanate)-Actidione-Natriumazid (i.e., sodium azide)-Egg yolk-Pyruvate (KRA-NEP) agar (Sinell and Baumgart, 1966), and Schleifer-Krämer (SK) agar (Schleifer and Krämer, 1980) for the selective isolation and enumeration of staphylococci from foods.

SK Agar for Selective Isolation of Staphylococci (Schleifer and Krämer, 1980)

Basal medium:	
Tryptone or peptone from casein	10.0 g
Beef extract	5.0 g
Yeast extract	3.0 g
Glycerol	10.0 g
Sodium pyruvate	10.0 g
Glycine	0.5 g
KSCN	2.25 g
$NaH_2PO_4 \cdot H_2O$	0.6 g
$Na_2HPO_4 \cdot 2H_2O$	0.9 g
LiCl	2.0 g
Agar	13.0 g
Distilled H_2O	1 liter

Adjust pH to 7.2. Autoclave at 121°C for 15 min, cool down in water bath to 45°C and add 10 ml of a 0.45% sterile-filtered solution of sodium azide. Mix medium thoroughly and pour immediately into Petri dishes. The medium can be stored at 4°C for at least one week. Staphylococci can be detected in various foods at levels as low as 100 colony-forming-units (CFU)/g of food. As in the case of KRANEP agar, the addition of egg yolk or pork plasma to the basal medium can provide a basis for distinguishing *S. aureus* from the coagulase-negative staphylococci.

The recovery of some animal species (e.g., *S. caprae* and *S. chromogenes*) can be improved by adding 5% sheep blood and/or reducing the level of sodium azide in SK agar from 45 to 15 mg/liter (Harvey and Gilmour, 1988).

Isolation of Staphylococci from Clinical Specimens

The isolation and enumeration of staphylococci from clinical specimens are routine operations in the hospital and veterinary clinical laboratory. Procedures for handling specimens and isolating and enumerating staphylococci can be found in the following texts: American Society for Micro-

biology (ASM) *Manual of Clinical Microbiology*, seventh edition (Murray et al., 1999), *A Guide to Specimen Management in Clinical Microbiology*, second edition (Miller, 1998) and *Clinical Microbiology Procedures Handbook* (Isenberg, 1994).

Staphylococci from a variety of clinical specimens are usually isolated in primary culture on blood agar and in a fluid medium such as thioglycolate broth. A general discussion of the preparation of specimens for primary culturing and inoculation of media and colony isolation methodology can be found in the *ASM Manual of Clinical Microbiology* (Murray et al., 1999).

ISOLATION AND CULTURE FROM CLINICAL SPECIMENS (KLOOS AND BANNERMAN, 1999) Every specimen should be plated onto blood agar (preferably sheep blood agar) and other media as indicated. On blood agar, abundant growth of most staphylococcal species occurs within 18–24 h. Since most species cannot be distinguished from one another during this time period, colonies should be picked at this time only for preliminary identification testing (e.g., when specimens are taken from patients with acute infections). Colonies should be allowed to grow for at least an additional two to three days before the primary isolation plate is confirmed for species or strain composition (Kloos and Schleifer, 1975b; Kloos and Schleifer, 1975c). This growth period is particularly important when the sampling of more than one colony is needed to obtain sufficient inocula for determining the predominant organism or to obtain a pure culture. Failure to hold plates for 72 h, can result in 1) selection of more than one species or strain if sampling yields an inoculum of two or more colonies, 2) selection of an organism(s) not producing the infection, if the specimen contains two or more different species or strains, and 3) incorrectly labeling a mixed culture as a pure culture. Colonies should be Gram-stained, subcultured, and tested for genus, species, and strain properties. It should be noted that most staphylococci of major medical interest produce growth in the upper as well as the lower anaerobic portions of thioglycolate broth or semisolid agar (Kloos and Schleifer, 1975c).

Fecal specimens suspected of containing infecting staphylococci (e.g., associated with staphylococcal enterocolitis) and other specimens from potentially heavily contaminated sources should also be inoculated on a selective medium such as SK agar (described above), Columbia CNA agar, lipase-salt-mannitol agar (LSM; Remel, Lenexa, KS, USA), tellurite glycine agar, phenylethyl alcohol agar, or mannitol salt agar. These media inhibit the growth of Gram-negative bacteria in addition to some other contaminating species. Incubation of these cultures should be for at least 48–72 h for discernible colony development.

BLOOD CULTURES AND CATHETER TIPS Blood cultures are usually indicated when there is a sudden increase in the pulse rate and temperature of the patient, a change in sensorium, and the onset of chills, prostration, and hypotension. Timing of collection is usually not critical when bacteremia is expected to be continuous; however, bacteremia often is intermittent. In these cases, timing may be very important, and bacteremia may precede the onset of fever or chill by as much as 1 h. Staphylococci are one of the major groups of bacteria that can produce a serious bacteremia.

Ideally, blood specimens should be collected before administration of antimicrobial agents and as close as possible to a fever spike. Blood for culture should be collected aseptically, first by cleaning the venipuncture site with 70% alcohol and then with an iodine preparation in a concentric fashion. The iodine should be allowed to dry. The venipuncture site should not be touched after cleansing. After venipuncture, the iodine should be removed from the skin with alcohol. It is recommended that 10–20 ml/set be collected in adults and 1–10 ml/set in infants. Also for acute sepsis, 2 or 3 sets should be collected from separate sites all within 10 min; for acute endocarditis, 3 sets should be collected from separate sites within 1–2 h; and for subacute endocarditis, 3 sets should be collected from separate sites taken 15 min apart. Initial processing of a blood specimen depends on the culture system used. All bacterial blood cultures should be incubated at 35°C for up to 7 days for evidence of growth. Gram-stained smears and subcultures of suspected positive cultures should be prepared immediately.

Culture of catheter tips may be performed to determine the source of bacteremia. The most commonly used method is the semi-quantitative method (Maki et al., 1977). A 5-cm segment of the distal tip is rolled across a blood agar plate four times. Cultures yielding > 15 bacterial colonies are considered clinically significant for a catheter-related infection.

PREPARATION OF BODY FLUID CULTURES Staphylococci may infect a variety of body fluids in addition to blood, such as cerebrospinal fluid (CSF) and joint, intraocular, pericardial, peritoneal, and pleural space fluids. It usually is easiest to establish the etiological agent in infections of normally sterile body sites, provided puncture and handling of the specimen are performed under conditions of strict asepsis. The skin

should be disinfected with a 2% solution of tincture of iodine. The specimen should be injected immediately into a sterile (screw-cap) tube or bottle. The specimen should be transported immediately to the laboratory for testing. Since only a small number of microorganisms may be present in clear or slightly cloudy fluids, volumes > 1 ml should be centrifuged to concentrate the organisms. A portion of the sediment is used for Gram staining and to inoculate the media.

PREPARATION OF URINE CULTURES Staphylococcal urinary tract infections in humans are commonly caused by *S. saprophyticus*, *S. epidermidis* and *S. aureus*. Acceptable methods for urine collection include midstream clean catch, catheterization, and suprapubic aspiration. At least 1 ml of midstream urine should be collected in a sterile, wide-mouth container. Unpreserved specimens should be cultured within 2 h of collection or stored in a refrigerator for no more than 24 h. Most references to diagnostic criteria state that a significant bacteriuria occurs when there are 100,000 cells or more per ml in a clean-voided, midstream specimen obtained from asymptomatic patients. With acute dysuria and frequency in young, sexually active females, a colony count as low as 100 per ml may be a useful criterion. Many significant urinary tract infections due to *S. saprophyticus* are associated with only 100 to 10,000 colonies per ml. If significant, these low counts may be substantiated by repetition of the procedure. Significant bacteriuria may also be determined by microscopic examination of a Gram-stained smear of uncentrifuged urine. The presence of at least two bacteria per 1000-X microscopic field of the Gram-stained smear is approximately equal to 100,000 or more cells per ml (Pollock, 1983).

ISOLATION OF STAPHYLOCOCCI FROM SKIN AND MUCOUS MEMBRANES Several basic methods are available for isolating staphylococci and other aerobic bacteria from skin and the adjacent mucous membranes (reviewed by Noble and Somerville, 1974). Washing or swabbing methods disperse cutaneous bacteria to provide samples of uniform composition. They break up large aggregates or microcolonies on skin into smaller colony-forming units (CFU) and in some cases single cells. Impression methods estimate the number of microcolonies or aggregates of bacteria on the skin surface. Biopsy methods can determine the location of bacteria in microniches on skin. Most of the sampling of aerobic bacteria on skin and mucous membranes has been performed using scrubbing and swabbing methods. The swab technique described by Kloos and

Musselwhite (1975a) is suitable for use with human as well as other mammalian skin. The medium most widely used for the isolation and culture of natural populations of staphylococci is P agar (Kloos et al., 1974).

P Agar

Peptone	10.0 g
Yeast extract	5.0 g
Sodium chloride	5.0 g
Glucose	1.0 g
Agar	15.0 g
Distilled water	1 liter

Adjust pH to 7.5 before autoclaving at 121°C for 15 min.

From each inoculated swab, a series of dilutions are prepared and plated on standard size (15 × 100 mm) P agar plates, in an attempt to obtain 50–300 isolated colonies on a plate for identification and enumeration. Inoculated plates are incubated at 34–35°C for 3–4 days and then held at room temperature for an additional 2 days. Each colony type is enumerated and one or two representatives of each type per plate are examined further for distinguishing genus, species, and strain characteristics. Colony morphology can be a useful supplementary character in the identification of species and strains. Selective media may be used in addition to the nonselective P agar if bacterial and/or fungal populations are very large (e.g., from the human inguinal and perineal area or from the skin of certain animals) and/or if the cutaneous flora contains species producing large, spreading colonies. Pigment production of colonies may be enhanced by the addition of milk, fat, glycerol monoacetate, or soaps to P agar or heart infusion agar (Willis et al., 1966).

Isolation of Staphylococci from Water

The presence of potentially pathogenic staphylococci in recreational waters, swimming pools, water that might be added to foods, and hydrotherapy pools poses a threat to human health (reviewed by Evans, 1977). Most attention is focused on the presence in water of *S. aureus* although several other staphylococcal species are also opportunistic pathogens. Staphylococci are somewhat resistant to halogen disinfectants. For this reason, significant numbers of these organisms can remain viable for extended periods of time in inadequately treated bathing places. Methods for the recovery and enumeration of pathogens from water are described in the manual jointly published by the American Public Health Association (APHA) and Water Environment Federation entitled *Standard Methods for the Examination of Water and Wastewater*, twentieth edition (Clesceri et al., 1998).

Phylogeny

On the basis of comparative 16S rRNA sequence studies, the genera *Staphylococcus* and *Macrococcus* belong to the Gram-positive bacteria with a low DNA G+C content. They are closely related to bacilli and other Gram-positive bacteria with low DNA G+C content such as enterococci, streptococci, lactobacilli and listeria (Fig. 1). Combining *Staphylococcus*, *Gemella*, *Macrococcus* and *Salinicoccus* within the family Staphylococcaceae has been proposed (Garrity and Holt, 2001).

The genera *Staphylococcus* and *Macrococcus* are monophyletic (Fig. 2) and well separated from each other with intergenera 16S rRNA sequence similarities of 93.4–95.3%. The intragenus similarities are significantly higher with at least 96.5% for staphylococci and 97.7% for macrococci. On the basis of DNA-DNA hybridization studies, staphylococcal species can be grouped (Kloos et al., 1992). The most important ones are the two coagulase-negative and novobiocin-susceptible species groups *S. epidermidis* (e.g., *S. capitis*, *S. caprae*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. saccharolyticus* and *S. warneri*) and *S. simulans* (e.g., *S. carnosos* and *S. simulans)*; the two coagulase-negative and

novobiocin-resistant species groups *S. saprophyticus* (e.g., *S. cohnii*, *S. saprophyticus* and *S. xylosus*) and *S. sciuri* (e.g., *S. lentus*, *S. sciuri* and *S. vitulus*), and the two coagulase-positive and novobiocin-susceptible species groups *S. intermedius* (e.g., *S. delphini* and *S. intermedius*) and *S. aureus* (e.g., *S. aureus* and *S. aureus* subsp. *anaerobius*).

On the basis of DNA-DNA hybridization studies, the species *M. equipercicus*, *M. bovicus* and *M. carouselicus* were more closely related to one another than to *M. caseolyticus* (Kloos et al., 1998a). Both DNA-DNA hybridization studies and 16S rRNA sequence analysis indicate a closer relationship of the genus *Macrococcus* to the *S. sciuri* species group than to other staphylococcal species.

Identification

General Properties

Members of the genus *Staphylococcus* are Gram-positive cocci (0.5–1.5 μm in diameter) that occur singly, in pairs, tetrads, short chains (three or four cells), and irregular grape-like clusters. They are nonmotile, nonsporeforming, and usually are unencapsulated or have limited capsule formation. Most species are facultative anaerobes and they are positive for the catalase and benzidine tests. With the exception of *S. saccharolyticus* and *S. aureus* subsp. *anaerobius*, growth is more rapid and abundant under aerobic conditions. These exceptional staphylococci are also catalase-negative. Most species contain *a*- and *b*-type cytochromes. The exceptional species *S. lentus*, *S. sciuri* and *S. vitulus* contain *a*-, *b*- and *c*-type cytochromes. Menaquinones are unsaturated (normal). The G+C content of DNA is in the range of 30–39 mol%. The genome size is in the range of 2000–3000 kb (George and Kloos, 1994; Kloos et al., 1998b). Staphylococci are generally susceptible to lysostaphin (some species more than others), furazolidone and nitrofuran, and resistant to erythromycin and bacitracin at low levels. In the laboratory routine, rapid distinction of staphylococci from micrococci can be made by demonstrating the susceptibility of staphylococci to 200 μg of lysostaphin per ml and resistance to erythromycin at 0.04 μg per ml, plus the production of acid from glycerol (Schleifer and Kloos, 1975b) or, alternatively, demonstrating susceptibility of staphylococci to a 100 μg furazolidone disk and resistance to a 0.04-unit bacitracin disk (Baker, 1984). Furthermore, staphylococci, with the exceptions of *S. lentus*, *S. sciuri* and *S. vitulus* exhibit a negative reaction with the rapid modified oxidase test, whereas micrococci are positive for this test (Faller and Schleifer, 1981).
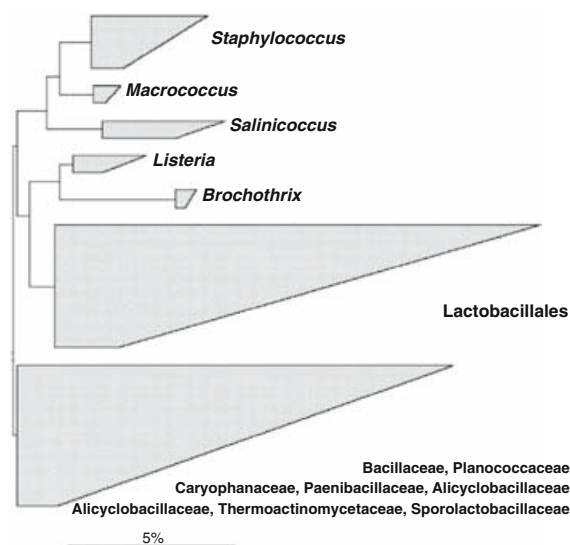
Fig. 1. A 16S rRNA-based tree reflecting the relationships of the *Staphylococcus-Macrococcus-Salinicoccus* cluster and its sister groups among the bacilli. The tree was reconstructed by a maximum likelihood analysis of all currently available almost complete (at least 80% with respect to the *E. coli* reference) small subunit rRNA sequences. Only alignment positions at which at least 50% of all included members share the same characters were included for tree calculation. The bar length indicates 5% estimated sequence divergence.
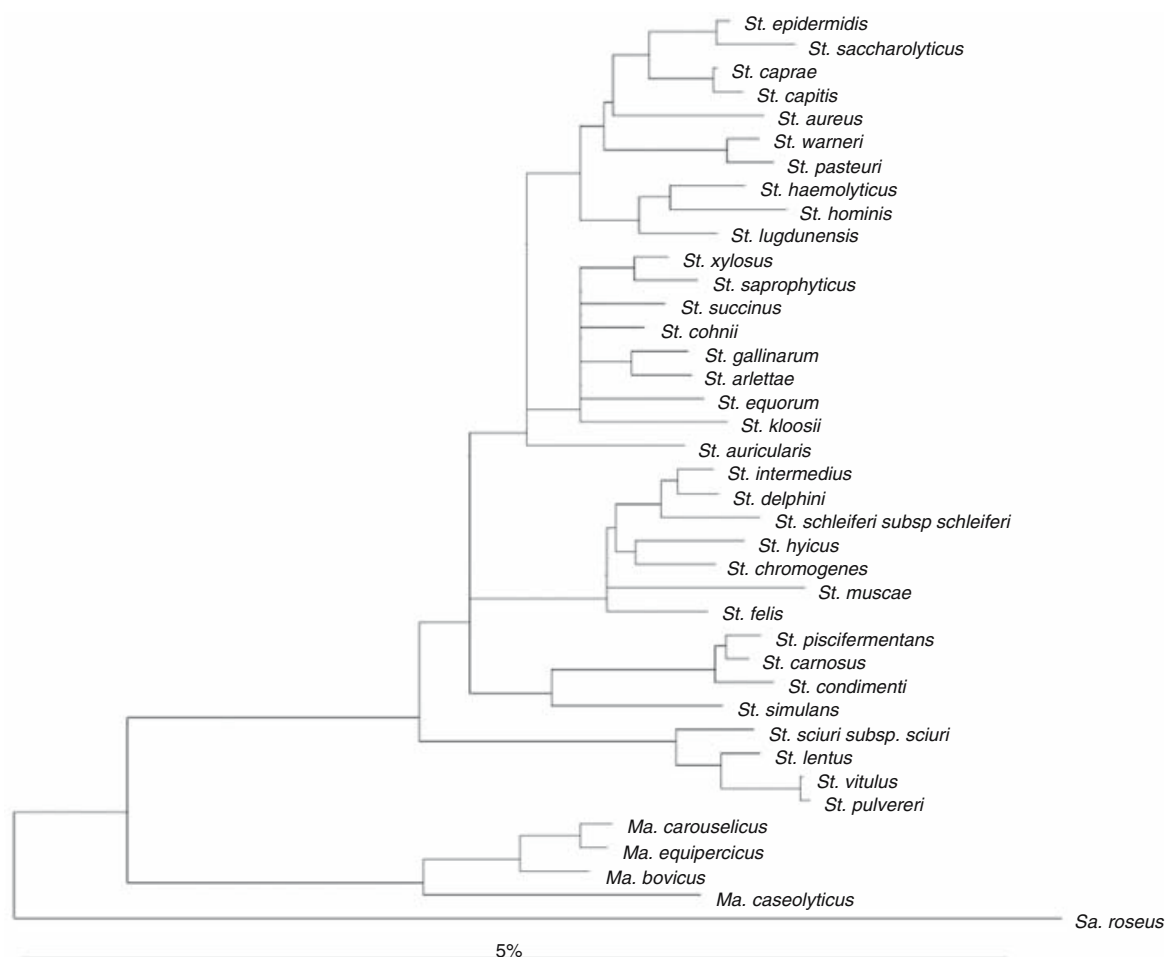
Fig. 2. A 16S rRNA-based tree reflecting the relationships of staphylococci, macrococci and salinicocci. The tree was reconstructed by a maximum likelihood analysis of all currently available almost complete (at least 80% with respect to the *E. coli* reference) small subunit rRNA sequences. Alignment positions at which the primary structures of less than 50% of all included members of the staphylococci, macrococci and salinicocci share a common character were excluded from the calculations. The tree topology was evaluated and corrected according to the results obtained by applying distance matrix and maximum likelihood approaches. (All available rRNA sequences from representatives of the Firmicutes and a selection of sequences representing the bacterial and archaeal phyla [1970 sequences] were used for distance matrix analyses, whereas the respective sequences from all staphylococci, macrococci and salinicocci in combination with a small selection of reference sequences of members of the sister groups [122 sequences] were analyzed by applying maximum likelihood procedures.) Multifurcations indicate that common relative branching order could not be unambiguously determined or was not supported when applying the alternative treeing approaches. The bar length indicates 5% estimated sequence divergence.

Members of the genus *Macrococcus* are Gram-positive cocci (1.1–2.5 μm in diameter) that occur mostly in pairs and tetrads, and occasionally single and arranged in short chains (Kloos et al., 1998a). They are nonmotile, nonsporeforming, and usually are unencapsulated. Macrococci are marginally facultative anaerobes; growth occurs better under aerobic conditions. They are positive for catalase and oxidase activities. They are resistant to bacitracin and lysozyme (25 μg/ml), and susceptible to furazolidone. Macrococci can contain *a*-, *b*- and/or *c*-type cytochromes. The G+C content of DNA is in the range of 38–45 mol%. The genome size is in the range of 1500–1800 kb.

Cell Wall Composition

The ultrastructure and chemical composition of the cell wall of staphylococci is similar to that of other Gram-positive bacteria. It consists of a thick (usually 60–80 nm), rather homogeneous, and not very electron-dense layer. It is made up of peptidoglycan, teichoic acid, and protein (Schleifer, 1983). Macrococci, with the possible

exception of *M. caseolyticus*, do not have detectable levels of teichoic acids.

A characteristic feature of the peptidoglycan of staphylococci is the occurrence of glycine-rich interpeptide bridges. Penta- and hexaglycine interpeptide bridges are found in about half of the staphylococcal species (peptidoglycan type: Lys-Gly$_{5-6}$). In most of the other half, a minor part of the glycine residues can be replaced with L-serine (peptidoglycan type: Lys-Gly$_4$, Ser). *Staphylococcus sciuri*, *S. lentus*, *S fleuretti* and *S. vitulus* may have an L-alanine instead of a glycine residue bound to lysine of the peptide subunit (peptidoglycan type: Lys-Ala-Gly$_4$). The peptidoglycan types for macrococci can be either Lys-Gly$_{3-4}$, L-Ser (*M. caseolyticus*, *M. equipercicus* and *M. carouselicus*) or Lys-Gly$_3$, L-Ser (*M. bovicus*).

Staphylococcal cell wall teichoic acids are water-soluble polymers containing repeating phosphodiester groups that are covalently linked to peptidoglycan. They consist of polyol (glycerol and ribitol) sugars and/or *N*-acetylamino sugars. Most staphylococci contain glycerol or ribitol teichoic acids. The teichoic acids consist of polymerized polyol phosphates that are substituted with various combinations of sugars and/or *N*-acetylamino sugar residues, and also ester-linked D-alanine residues. In some cases, *N*-acetylamino sugar residues can also form an integral part of the polymer chain (Endl et al., 1983; Endl et al., 1984). The occurrence of the same major components does not always mean that the structure of teichoic acid is identical; for example, the teichoic acids of *S. capitis* and *S. hyicus* show a similar composition but their structures are quite different.

Differentiation of Species and Subspecies

The classification of species and subspecies of staphylococci and macrococci can be based on a variety of phenotypic character analyses and DNA-DNA (genomic) relationships. In addition, rRNA analysis and ribotyping may be used to describe the relationship of reference and new species. DNA similarity (> 70%) is the criterion that has been used to determine species boundaries in the formal classification of staphylococcal species and most subspecies. Selected phenotypic characters are also useful in classification because they had a high predictive value in identifying DNA similarity groups. Recently, recommended minimal standards for the description of new staphylococcal species have been published (Freney et al., 1988). Characters studied at the cellular and population levels, including morphological and physiological properties, enzyme reactions, and intrinsic resistance to certain antibiotics, have been included in practical identification schemes as shown in Tables 2–5. Molecular studies of phenotypic characters have also provided a basis for determining epigenetic relationships.

CONVENTIONAL METHODS Conventional methods for the determination of phenotypic characters at the cellular and population levels were developed first and then examined for their correlation to DNA relatedness (reviewed by Kloos

Table 2. Differentiation of coagulase/clumping factor-positive *Staphylococcus* species and subspecies.

Character	*S. aureus*	*S. aureus* subsp. *anaerobius*	*S. delphini*	*S. hyicus*	*S. intermedius*	*S. lugdunensis*	*S. lutrae*	*S. sciuri* subsp. *carnaticus*	*S. sciuri* subsp. *rodentium*	*S. schleiferi*	*S. schleiferi* subsp. *coagulans*
Colony size ±6mm	+	−	+	+	+	d	−	−	d	−	d
Colony pigment	+	−	−	−	−	d	−	d	d	−	−
Anaerobic growth	+	(+)	(+)	+	(+)	+	+	(d)	(d)	+	+
Aerobic growth	+	()	+	+	+	+	+	+	+	+	+
Staphylocoagulase	+	+	+	d	+	−	+	−	−	−	+
Clumping factor	+	−	−	−	d	(+)	−	d	+	+	−
Thermonuclease	+	+	−	+	+	−	()	−	−	+	+
Hemolysis	+	+	+	−	d	(+)	+	()	()	(+)	(+)
Catalase	+	−	+	+	+	+	+	+	+	+	+
Modified oxidase	−	−	−	−	−	−	−	+	+	−	−
Alkaline phosphatase	+	+	+	+	+	−	+	d	d	+	+
Pyrrolidonyl arylamidase	−	ND	ND	−	+	+	ND	−	−	+	ND
Ornithine decarboxylase	−	ND	ND	−	−	+	ND	−	−	−	ND
Urease	d	ND	+	d	+	d	+	−	−	−	+

Table 2. *Continued*

Character	*S. aureus*	*S. aureus* subsp. *anaerobius*	*S. delphini*	*S. hyicus*	*S. intermedius*	*S. lugdunensis*	*S. lutrae*	*S. sciuri* subsp. *carnaticus*	*S. sciuri* subsp. *rodentium*	*S. schleiferi*	*S. schleiferi* subsp. *coagulans*
β-Glucosidase	+	−	ND	d	d	+	ND	+	+	−	ND
β-Glucuronidase	−	−	ND	+	−	−	ND	−	−	−	ND
β-Galactosidase	−	−	ND	−	+	−	+	−	−	(+)	ND
Arginine dihydrolase	+	ND	+	+	d	−	−	−	−	+	+
Acetoin production	+	−	−	−	−	+	−	−	−	+	+
Nitrate reduction	+	−	+	+	+	+	+	+	+	+	+
Esculin hydrolysis	−	−	ND	−	−	−	ND	+	+	−	ND
Novobiocin resistance	−	−	−	−	−	−	−	+	+	−	−
Acid (aerobically) from:											
D-Trehalose	+	−	−	+	+	+	+	+	(+)	d	−
D-Mannitol	+	ND	(+)	−	(d)	−	d	+	+	−	d
D-Mannose	+	−	+	+	+	+	+	(d)	(+)	+	+
D-Turanose	+	ND	ND	−	d	(d)	ND	ND	ND	−	ND
D-Xylose	−	−	−	−	−	−	+	+	(d)	−	−
D-Cellobiose	−	−	ND	−	−	−	ND	(d)	d	−	−
L-Arabinose	−	−	−	−	−	−	ND	d	(d)	−	−
Maltose	+	+	+	−	()	+	+	(d)	(d)	−	−
Sucrose	+	+	+	+	+	+	ND	+	+	−	d
N-Acetylglucosamine	+	−	ND	+	+	+	ND	−	−	(+)	ND
Raffinose	−	−	ND	−	−	−	ND	−	−	−	−

Symbols: +, 90% or more strains; −, 90% or more strains negative; d, 11 to 89% of strains positive; ND, not determined. () indicate a delayed reaction.

Adapted from Kloos and Bannerman (1999); Kloos and Scheifer (1986); Schleifer (1986).

Table 3. Differentiation of coagulase-negative, novobiocin-susceptible *Staphylococcus* species and subspecies.

Character	*S. auricularis*	*S. capitis*	*S. capitis* subsp. *ureolyticus*	*S. caprae*	*S. carnosus*	*S. carnosus* subsp. *utilis*	*S. chromogenes*	*S. condimenti*	*S. epidermidis*	*S. felis*	*S. haemolyticus*	*S. hominis*	*S. lugdunensis*	*S. muscae*	*S. pasteuri*	*S. piscifermentans*	*S. saccharolyticus*	*S. schleiferi*	*S. simulans*	*S. succinus*	*S. warneri*
Colony size ±6mm	−	−	(d)	d	+	−	+	ND	−	+	+	−	d	d	d	ND	−	−	+	ND	d
Colony pigment	−	−	(d)	−	−	−	+	d	−	−	d	d	−	d	d	−	−	−	−	−	d
Anaerobic growth	(+)	(+)	(+)	(+)	+	+	+	+	+	+	+	(+)	()	+	+	+	+	+	+	+	+
Aerobic growth	(+)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	()	+	+	+	+
Staphylocoagulase	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	ND	−
Clumping factor	−	−	−	−	−	ND	−	ND	−	−	−	−	(+)	−	−	ND	−	+	−	ND	−
Thermonuclease	−	−	−	−	−	ND	−	ND	−	−	−	−	−	−	−	−	−	+	−	ND	−
Hemolysis	−	(d)	(d)	(d)	−	ND	−	ND	(d)	(d)	(+)	−	(+)	(+)	(d)	−	−	(+)	(d)	ND	(d)
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
Modified oxidase	−	−	−	−	−	ND	−	ND	−	−	−	−	−	−	−	−	−	−	−	−	−
Alkaline phosphatase	−	−	−	(+)	+	−	+	+	+	+	−	−	−	+	−	+	d	+	(d)	+	−
Pyrrolidonyl arylamidase	d	−	(d)	d	+	ND	d	ND	−	ND	+	−	+	ND	−	ND	ND	+	+	−	−
Ornithine decarboxylase	−	−	−	−	−	ND	−	ND	(d)	ND	−	−	+	−	−	ND	−	−	−	ND	−
Urease	−	−	+	+	−	−	+	+	+	+	−	+	d	−	+	+	ND	−	+	+	+
β-Glucosidase	−	−	−	−	−	ND	d	ND	(d)	−	d	−	+	ND	+	+	ND	−	−	ND	+
β-Glucuronidase	−	−	−	−	−	−	−	−	−	−	d	−	−	ND	+	−	ND	−	d	ND	d
β-Galactosidase	(d)	−	−	−	+	−	−	+	−	+	−	−	−	−	−	−	ND	(+)	+	ND	−
Arginine dihydrolase	d	d	+	+	+	+	+	+	d	+	+	d	−	−	d	+	+	+	+	−	d
Acetoin production	−	d	d	+	+	ND	−	ND	+	−	+	d	+	−	d	−	ND	+	d	−	+

Table 3. *Continued*

Character	S. auricularis	S. capitis	S. capitis subsp. urealyticus	S. caprae	S. carnosus	S. carnosus subsp. utilis	S. chromogenes	S. condimenti	S. epidermidis	S. felis	S. haemolyticus	S. hominis	S. lugdunensis	S. muscae	S. pasteuri	S. piscifermentans	S. saccharolyticus	S. schleiferi	S. simulans	S. succinus	S. warneri
Nitrate reduction	(d)	d	+	+	+	d	+	+	+	+	+	d	+	ND	d	+	+	+	+	–	d
Esculin hydrolysis	–	–	–	–	–	–	–	–	–	ND	–	–	–	ND	–	+	ND	–	–	ND	–
Novobiocin resistance	–	–	–	–	–	ND	–	ND	–	–	–	–	–	–	–	–	–	–	–	ND	–
Acid (aerobically) from:																					
D-Trehalose	(+)	–	–	(+)	d	d	+	+	–	+	+	d	+	+	+	+	–	d	d	+	+
D-Mannitol	–	+	+	d	+	–	d	+	–	+	d	–	–	d	d	–	–	+	–	ND	d
D-Mannose	–	+	+	+	+	–	+	+	(+)	+	–	–	+	–	–	–	(+)	+	d	+	–
D-Turanose	(d)	–	–	–	–	d	–	d	(d)	ND	(d)	+	(d)	+	(d)	–	ND	–	–	ND	(d)
D-Xylose	–	–	–	–	–	ND	–	ND	–	–	–	–	–	+	–	–	–	–	–	ND	–
D-Cellobiose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–
L-Arabinose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–
Maltose	(+)	–	+	(d)	–	–	d	–	+	–	+	+	+	–	(d)	d	–	–	()	ND	(+)
Sucrose	d	(+)	+	–	–	–	+	–	+	d	+	(+)	+	+	+	d	–	–	+	ND	+
N-Acetylglucosamine	–	–	–	–	ND	ND	d	ND	–	+	+	d	+	ND	ND	ND	ND	(+)	+	ND	–
Raffinose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	(d)	–

Symbols: +, 90% or more strains; –, 90% or more strains negative; d, 11 to 89% of strains positive; ND, not determined. () indicate a delayed reaction.

Adapted from Kloos and Bannerman (1999); Kloos and Scheifer (1986); Schleifer (1986).

Table 4. Differentiation of novobiocin-resistant *Staphylococcus* species and subspecies.

Character	S. arlettae	S. cohnii	S. cohnii subsp. urealyticus	S. equorum	S. fleurettii	S. gallinarum	S. hominis subsp. novobiosepticus	S. kloosii	S. lentus	S. saprophyticus	S. saprophyticus subsp. bovis	S. sciuri	S. sciuri subsp. carnaticus	S. sciuri subsp. rodentium	S. xylosus	S. vitulinus
Colony size ±6mm	d	d	+	–	–	+	–	d	–	+	–	+	–	d	+	–
Colony pigment	+	–	d	–	–	d	–	d	d	d	+	d	d	d	d	+
Anaerobic growth	–	d	(+)	–	+	(+)	–	–	()	(+)	+	(+)	(d)	(d)	d	–
Aerobic growth	+	+	+	(+)	+	+	+	+	(+)	+	+	+	+	+	+	(+)
Staphylocoagulase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Clumping factor	–	–	–	–	ND	–	–	–	–	–	–	–	–	d	+	–
Thermonuclease	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Hemolysis	–	(d)	(d)	(d)	ND	(d)	–	(d)	–	–	–	()	()	()	–	–
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Modified oxidase	–	–	–	–	+	–	–	–	+	–	–	+	+	+	–	+
Alkaline phosphatase	(+)	–	+	(+)	d	(+)	–	d	()	–	–	+	d	d	d	–
Pyrrolidonyl arylamidase	–	–	d	–	–	–	–	d	–	–	+	–	–	–	d	–
Ornithine decarboxylase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Urease	–	–	+	+	–	+	+	d	–	+	+	–	–	–	+	–
β-Glucosidase	ND	–	–	ND	ND	+	–	d	+	d	d	+	+	+	+	d
β-Glucuronidase	+	–	+	+	–	d	–	d	–	–	–	–	–	–	+	–
β-Galactosidase	d	–	+	d	–	d	–	d	–	+	d	–	–	–	+	–
Arginine dihydrolase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Acetoin production	–	d	d	–	d	–	d	d	–	+	d	–	–	–	d	–
Nitrate reduction	–	–	–	+	+	+	d	–	+	–	+	+	+	+	d	+
Esculin hydrolysis	–	–	–	d	d	+	–	d	+	–	–	+	+	+	d	d

(*Continued*)

Table 4. *Continued*

Character	S. arlettae	S. cohnii	S. cohnii subsp. urealyticus	S. equorum	S. fleurettii	S. gallinarum	S. hominis subsp. novobiosepticus	S. kloosii	S. lentus	S. saprophyticus	S. saprophyticus subsp. bovis	S. sciuri	S. sciuri subsp. carnaticus	S. sciuri subsp. rodentium	S. xylosus	S. vitulinus
Acid (aerobically) from:																
D-Trehalose	+	+	+	+	+	+	–	+	+	+	+	+	+	(+)	+	(d)
D-Mannitol	+	d	+	+	ND	+	–	+	+	d	+	+	+	+	+	+
D-Mannose	+	(d)	+	+	+	+	–	–	(+)	–	–	(d)	(d)	(+)	+	–
D-Turanose	+	–	–	d	+	+	ND	–	()	+	+	()	ND	ND	d	–
D-Xylose	+	–	–	+	d	+	–	(d)	()	–	–	(d)	+	(d)	+	d
D-Cellobiose	–	–	–	(d)	–	+	–	–	+	–	–	+	(d)	d	–	d
L-Arabinose	+	–	–	+	d	+	–	d	d	–	–	d	d	(d)	d	–
Maltose	+	(d)	(+)	d	+	+	+	d	d	+	+	(d)	(d)	(d)	+	–
Sucrose	+	–	–	+	+	+	(+)	()	+	+	+	+	+	+	+	+
N-Acetylglucosamine	–	–	d	d	ND	+	–	–	d	d	+	d	–	–	+	–
Raffinose	+	–	–	–	–	+	–	–	+	–	–	–	–	–	–	–

Symbols: +, 90% or more strains; –, 90% or more strains negative; d, 11 to 89% of strains positive; ND, not determined. () indicate a delayed reaction.
Adapted from Kloos and Bannerman (1999); Kloos and Scheifer (1986); Schleifer (1986).

Table 5. Conventional identification methods useful for the differentiation of *Macrococcus* species.

Character	M. caseolyticus	M. equipercicus	M. bovicus	M. carouselicus	S. sciuri subsp. sciuri	S. sciuri subsp. carnaticus	S. sciuri subsp. rodentium	S. lentus	S. fleuretii	S. vitulus
Anaerobic growth	d	–	–	–	(+)	(d)	(d)	()	+	–
Heat-stable nuclease	d	–	d	+	–	–	–	–	–	–
Alkaline phosphatase	–	–	–	–	+	d	d	()	d	–
Pyrrolidonyl arylamidase	+	–	–	–	–	–	–	–	–	–
Urease	–	d	d	–	–	–	–	–	–	–
β-Glucosidase	–	–	d	–	+	+	+	+	ND	d
Acetoin production	+	–	–	–	–	–	–	–	d	–
Nitrate reduction	+	–	–	–	+	+	+	+	+	+
Esculin hydrolysis	d	d	–	+	+	+	+	+	d	d
Acid (aerobically) from:										
D-Mannitol	–	+	+	d	+	+	+	+	ND	+
D-Mannose	–	–	–	–	(d)	(d)	(+)	(+)	+	–
D-Turanose	–	–	–	–	()	ND	ND	()	+	–
D-Xylose	–	–	–	–	(d)	+	(d)	()	d	d
D-Cellobiose	–	–	–	–	+	(d)	d	+	–	d
L-Arabinose	–	–	–	–	d	d	(d)	d	d	–
Maltose	+	d	d	–	(d)	(d)	(d)	d	+	–
Sucrose	d	–	d	d	+	+	+	+	+	+
Raffinose	–	–	–	–	–	–	–	+	–	–

Symbols: +, 90% or more of strains positive; –, 90% or more strains negative; d, 11 to 89% of strains positive; ND, not determined. () indicate a delayed reaction.

and Schleifer [1986] and Schleifer [1986]). Key characters now used for species and subspecies identification include the following: colony morphology, oxygen requirements, coagulase, clumping factor, heat-stable nuclease (thermonuclease), hemolysins, catalase, oxidase, alkaline phosphatase, urease, ornithine decarboxylase, pyrrolidonyl arylamidase, β-galactosidase, acetoin production, nitrate reduction, esculin hydrolysis, aerobic acid production from a variety of carbohydrates including D-trehalose, D-mannitol, D-mannose, D-turanose, D-xylose, D-cellobiose, L-arabinose, maltose, α-lactose, sucrose, and raffinose, and intrinsic resistance to novobiocin and polymyxin B (reviewed by Kloos and Bannerman, 1999). Some conventional methods may require up to three to five days before a final result can be obtained, while others only require several hours for interpretation. They are usually quite reliable and have served as a reference for more recent studies aimed at simplifying and expediting character analyses.

Rapid Identification System To facilitate identification in the routine or clinical laboratory, several manufacturers have developed rapid species identification kits or automated systems requiring only a few hours to one day for the completion of tests. Identification of a number of the *Staphylococcus* species can be made with an accuracy of 70 to > 90% using the commercial systems (Kloos and Bannerman, 1994). Since their introduction, these systems have been improved and expanded to include more species. Their reliability will continue to increase as the result of a growing database and the addition of more discriminating tests. *Staphylococcus aureus*, *S. epidermidis*, *S. capitis*, *S. haemolyticus*, *S. saprophyticus*, *S. simulans* and *S. intermedius* can be identified reliably by most of the commercial systems now available. For some systems, reliability depends upon additional testing as suggested by the manufacturer and/or by published evaluations of the product. Some identification systems now available include the following: RAPIDEC Staph (for identification of *S. aureus*, *S. epidermidis* and *S. saprophyticus*) and API STAPH (bioMérieux Vitek, Inc., Hazelwood, MO, USA); VITEK, a fully automated microbiology system that uses a Gram-positive identification (GPI) card (bioMérieux Vitek); MicroScan Pos ID panel (read manually or on MicroScan instrumentation) and MicroScan Rapid Pos ID panel (read by the WalkAway systems; in addition, the ID panels are available with antimicrobial agents for susceptibility testing; Dade MicroScan, Inc., West Sacramento, CA, USA); Crystal Gram-Positive Identification System, Crystal Rapid Gram-Positive Identification System, Pasco MIC/ID Gram-Positive Panel, and the Phoenix, an automated identification system (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA); GP MicroPlate test panel (read manually, using Biolog MicroLog system, or automatically with the Biolog MicroStation system; Biolog, Haywood, CA, USA); MIDI Sherlock Identification System Microbial Identification System (MIS) that automates microbial identification by combining cellular fatty acid analysis with computerized high-resolution gas chromatography (MIDI, Newark, DE, USA); and RiboPrinter Microbial Characterization System (Qualicon, Inc. Wilmington, DE, USA), based on ribotype pattern analysis.

Rapid detection of the species *S. aureus* can be made using the AccuProbe culture identification test for *S. aureus* (Gen-Probe, Inc., San Diego, CA, USA). This test is a DNA probe assay directed against rRNA, and it is very accurate (100% specificity). Tube coagulase-negative and slide test-negative strains of *S. aureus* should be identified correctly by the AccuProbe test. Additionally, PCR analysis of the 16S–23S rRNA intergenic spacer region has preliminarily shown successful results in discriminating among 31 *Staphylococcus* species (Mendoza et al., 1998). PCR analysis allows for the identification of pure culture staphylococci within 24–48 h.

Physiology

Sugar Transport

Sugar transport, metabolism, and catabolite repression of staphylococci is best studied in the pathogenic species *S. aureus* and the nonpathogenic species *S. xylosus*. Two types of carbohydrate transport have been identified and studied in staphylococci: 1) the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS), which is responsible for the binding, transmembrane transport, and phosphorylation of numerous sugar substrates, and 2) the PTS-independent carbohydrate transport in which the sugar is transported via a permease and is subsequently phosphorylated by an ATP-dependent kinase.

The known sugar transport systems in *S. aureus* and *S. xylosus* are summarized in Table 6. The type of transport used depends on the sugar and staphylococcal species. For some sugars, e.g., glucose, both types of transport systems are present in one species to ensure efficient transport, and they are functional under specific environmental conditions. Studies with PTS-deficient strains of *S. aureus* (Reizer et al., 1988), for example, have demonstrated the utilization of glucose by both PTS-dependent and PTS-independent mechanisms.

The Phosphotransferase System (PTS) The PTS delivers exogenous carbohydrates as phos-

Table 6. Carbohydrate transport systems in *S. aureus* and *S. xylosus*.

Carbohydrate	*S. aureus*		*S. xylosus*	
	PTS	PTS-independent	PTS	PTS-independent
Glucose	+	+	+	+
Galactose	+	ND	−	+
Fructose	ND	ND	+	ND
N-Acetylglucosamine	+	ND	ND	ND
Mannitol	+	ND	+	ND
Mannose	+	ND	ND	ND
Trehalose	ND	ND	+	ND
Lactose	+	ND	−	+
Maltose	ND	ND	+	ND
Sucrose	ND	ND	+	ND
Xylose	ND	−	−	+
Ribose	ND	ND	−	+
Arabinose	ND	−	−	+

Symbols and abbreviations: +, described transporters; −, not present; PTS, phosphoenolpyruvate (PEP): sugar phosphotransferase system; and ND, not determined.

phate esters to the cell cytoplasm. It consists of two nonspecific, energy-coupling components, enzyme I (EI, *ptsI*) and a heat-stable phospho-carrier protein (HPr, *ptsH*), as well as several sugar-specific multiprotein permeases known as enzymes II (EIIA, B and C). In most cases, enzymes IIA and IIB are located in the cytoplasm, while enzyme IIC forms a membrane channel.

EI and HPr play a central role in the PTS-mediated uptake of most sugars; both enzymes are soluble. The 80-kDa EI of *S. aureus* has been partially purified and appears to be monomeric. The EI protein is phosphorylated by PEP, a reaction that requires Mg^{+2}. The phosphorylation occurs at a histidine residue in the N3 position. HPr is a small protein with a M_r of 8300; its amino acid sequence has been determined (Bey-reuther et al., 1977). Also, HPr is phosphorylated by EI-P in position N1 of His-15 (Schrecker et al., 1975). A comparison of the primary structures of HPr proteins of various Gram-positive and -negative bacteria revealed a similar size and three highly conserved centers (Reizer et al., 1988).

GLUCOSE-SPECIFIC PTS The data available on staphylococcal PTS-dependent glucose uptake are restricted to *S. carnosus*. Two genes located next to each other, *glcA* and *glcB*, have been cloned; the genes complement an *Escherichia coli* mutant strain deficient in glucose uptake (Christiansen and Hengstenberg, 1996). The GlcA and GlcB proteins are highly similar to each other (69% identity) and to glucose-specific enzyme II proteins from *Bacillus subtilis* and *E. coli*.

The two *S. carnosus* PTS EII permeases have fused EII domains in the order EIICBA. Glu-cose is the primary substrate of both permeases, but various glucosides may also be recognized (Christiansen and Hengstenberg, 1999). The gene *glcT* is immediately upstream of *glcA* and the deduced amino acid sequence of its protein GlcT shows a high degree of similarity to bacterial regulators involved in antitermination (Stülke et al., 1998). Interestingly, the activity of these regulators is controlled by PTS-mediated phosphorylation. A putative transcriptional terminator partially overlapping an inverted repeat, which could be the target site for the antiterminator protein GlcT, is found in the *glcT*-*glcA* intergenic region. This organization resembles the *glcT*-*ptsG* region of *B. subtilis*, encoding the GlcT antiterminator protein and the glucose-specific enzyme II, respectively (Stülke and Hillen, 1999). Therefore, *glcA* expression in *S. carnosus* is most likely controlled by antitermination. Studies of *S. carnosus* GlcT activity in the heterologous host *B. subtilis* have indicated that the protein is indeed able to cause antitermination (Knezevic et al., 2000).

In *S. aureus* as well as in *S. xylosus*, genes encoding glucose-specific PTS permeases are not arranged in tandem. *Staphylococcus carnosus* may therefore be an exception among the staphylococci. As already mentioned, glucose uptake does not solely rely on PTS activity since HPr or EI mutants of *S. aureus* still ferment glucose. The same is also true for *S. xylosus* (Jankovic and Brückner, 2002) and *S. carnosus* (Brückner, 1997); the entry of glucose into the cells by two different mechanisms may thus be a general phenomenon.

LACTOSE-SPECIFIC PTS The EIIA^{lac} protein of the lactose-specific PTS of *S. aureus* consists of 103 amino acids (Stüber et al., 1985). Each of the

three subunits is phosphorylated via HPr-P at the N3 position of His-82 (Sobek et al., 1984). The lactose-specific enzyme EIICB[lac] of *S. aureus* couples translocation to phosphorylation of the transported lactose. It is composed of the N-terminal membrane-bound IIC domain, which includes the sugar-binding site, and the C-terminal IIB domain, which contains the phosphorylation site at Cys-476 (Peters et al., 1995). The kinetics of various EIICB[lac] fusion constructs have been investigated (Kowolik and Hengstenberg, 1998). The protein, which normally functions as a trimer, is believed to separate into its subunits after phosphorylation. Some of its structural features, like the presence of two histidine residues at the active site, seem to be common to all enzymes, although there is no overall structural similarity to any PTS proteins or to any other proteins in the Protein Data Bank (Sliz et al., 1997). Relevant PTS genes annotated in the genome of *S. aureus* N315 are shown in Table 7.

PTS-INDEPENDENT SUGAR TRANSPORT Only little is known about PTS-independent sugar transport in *S. aureus*. The situation is better with *S. xylosus*, in which sugar transport and its regulation has been investigated by the group of R. Brückner (Tübingen).

In *S. xylosus*, GlcU and GlkA constitute a glucose utilization system enabling glucose to be catabolized independently from the PTS. Glucose is transported through the cell membrane via GlcU in a nonphosphorylated form and is subsequently phosphorylated by GlkA. This PTS-independent system substantially contributes to glucose catabolism in *S. xylosus*. The novel, non-PTS glucose uptake gene *glcU* and the downstream gene *gdh*, which encodes glucose dehydrogenase, are found in a bicistronic operon (Fiegler et al., 1999). A similar organization has also been found in *B. subtilis*. The glucose kinase gene, *glkA*, is not part of the operon in *S. xylosus* (Wagner et al., 1995). The *glcU* and *glkA* genes were first detected by using Tn*917* insertions that resulted in a defect in glucose-specific catabolite repression of the lactose operon. Several other catabolic operons are also affected, which suggests the involvement of GlcU and GlkA in catabolite repression. There is evidence that the GlcU permease is functional at high extracellular glucose concentrations; the highly efficient glucose-specific PTS recognizes and transports glucose preferentially at low external glucose concentrations. The mechanism by which GlcU allows glucose to enter the cells remains to be elucidated. In the genome sequence of *S. aureus*, homologs of *glcU* and *glkA* are present, which strongly suggests that

Table 7. PTS and non-PTS sugar transporters of *S. aureus* N315 and *S. aureus* NCTC 8325.

Gene designation	Encoded protein
General PTS genes (organized in an operon)	
ptsH	Phosphocarrier protein HPr
ptsI	Phosphoenolpyruvate-protein phosphatase, enzyme I
Glucose-specific PTS genes	
ptsG	Glucose-specific IIABC component
glcA	Glucose-specific IIA homolog
—	Glucose-specific IIA homolog
Other sugar specific PTS genes	
mtlF	Mannitol-specific IIBC component
mtlA	Mannitol-specific IIA component
lacE	Lactose-specific IIBC component
lacF	Lactose-specific IIA component
scrA	Sucrose-specific IIBC component
ptaA	*N*-Acetylglucosamine-specific IIABC component
—	Maltose- and glucose-specific II homolog
—	Similar to fructose-specific IIBC
gatC	Similar to galactitol-specific IIB
—	Galactitol-specific enzyme IIC homolog
—	β-Glucoside-specific II ABC
glvC	Arbutin-like IIBC component
treP	Trehalose-specific enzyme II
Non-PTS transporters	
gntP	Gluconate permease
uhpT	Hexose phosphate transport protein
rbsD	Ribose permease
glpT	Glycerol-3-phosphate transporter

Symbol and abbreviation: PTS, phosphoenolpyruvate (PEP):sugar phosphotransferase system; and −, not yet assigned.
[a]Genome sequenced by Kuroda et al. (2001).
[b]Sequenced at the University of Oklahoma Health Sciences Center.

also in this and perhaps other staphylococcal species, glucose is taken up by both PTS-dependent and PTS-independent mechanisms.

Staphylococcus xylosus is unable to utilize arbutin, raffinose, cellobiose, sorbitol, rhamnose and fucose, and fermentation of salicin and ribose is very weak. Sucrose and fructose are transported exclusively via the PTS system, and PTS plays a central role in trehalose, mannose, maltose, maltotriose, and mannitol transport. Galactose and lactose (Bassias and Brückner, 1998), the pentoses xylose, ribose and arabinose, the corresponding pentitols, and glycerol are clearly non-PTS sugars (Lehmer and Schleifer, 1980; Table 6). The lactose utilization enzymes of *S. xylosus* comprise LacP (lactose permease) and LacH (β-galactosidase). The *lacR* gene, found upstream of *lacPH* encodes the activator (Bassias and Brückner, 1998). Lactose transport and β-galactosidase activity are induced by the addition of lactose to the growth medium. The

lacPH promoter is also subject to carbon catabolite repression mediated by the catabolite control protein CcpA. Relevant PTS-independent transporter genes annotated in the genome of *S. aureus* N315 are shown in Table 7.

THE GLYCOLYTIC PATHWAY Staphylococci are facultatively anaerobic microorganisms. The fructose 1,6-bisphosphate (FBP) and the oxidative

Table 8. Genes of the FBP pathway of *S. aureus* N315 and *S. aureus* NCTC 8325.

Gene designation	Encoded protein
—	Similar to glucokinase
pgi	Glucose-6-phosphate isomerase A
pfk	6-Phosphofructokinase
fbaA	Fructose-bisphosphate aldolase
tpi	Triosephosphate isomerase
gap	Glyceraldehyde-3-phosphate dehydrogenase
pgk	Phosphoglycerate kinase
pgm	2,3-Diphosphoglycerate-independent phosphoglycerate mutase
eno	Enolase (2-phosphoglycerate dehydrogenase)
pykA	Pyruvate kinase

Symbol and abbreviation: –, not yet assigned; and FBP, fructose 1,6-bisphosphate.
[a]Genome sequenced by Kuroda et al. (2001).
[b]Sequenced at the University of Oklahoma Health Sciences Center.

pentose phosphate (PP) pathways are the two central routes of glucose metabolism. There is no evidence for the existence of the Entner-Doudoroff pathway. Earlier studies of glucose metabolism, mainly confined to the oxidative aspects in *S. aureus*, are reviewed by Blumenthal (1972).

The *S. aureus* N315 genome sequence is available (Kuroda et al., 2001), allowing all genes of the FBP glycolytic pathway to be annotated. The genes are listed in the order of the enzymatic steps of the pathway in Table 8.

GALACTOSE AND LACTOSE METABOLISM (THE LELOIR AND THE TAGATOSE-6-P PATHWAYS) In many microorganisms, galactose is usually metabolized via the Leloir pathway (Bissett and Anderson, 1973; Bissett and Anderson, 1974). In *S. aureus*, galactose is converted to D-galactose 6-P, which is further metabolized through tagatose derivatives (Fig. 3). A study of the distribution of the tagatose-6-P pathway in various staphylococcal species has revealed that the key enzyme of this pathway, tagatose-6-P kinase, is found in *S. aureus*, *S. epidermidis* and *S. hominis*. These species do not possess enzymes of the Leloir pathway. The tagatose-6-P kinase is inducible by growth on galactose or lactose. In contrast, *S. intermedius*, *S. saprophyticus* and *S. xylosus* use galactose only via the Leloir pathway and not via the tagatose-6-P pathway (Schleifer
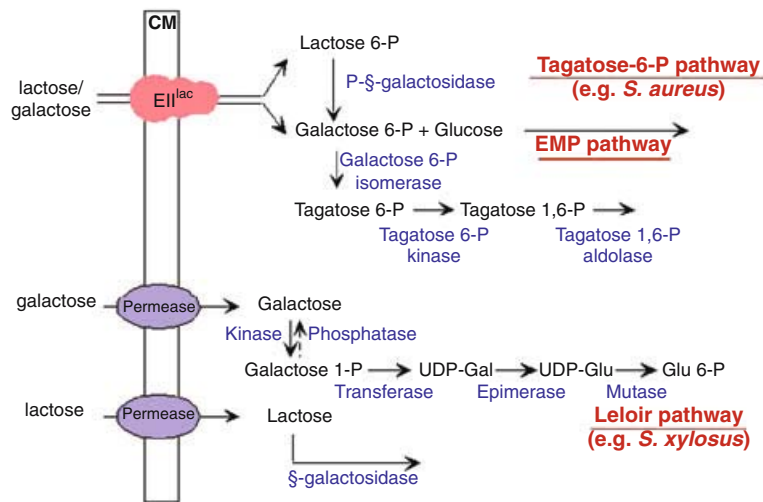
Fig. 3. Lactose and galactose pathways of staphylococci. Transport of lactose and galactose and their catabolism are shown. In *S. aureus*, lactose and galactose are transported by the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS). Internalized lactose 6-phosphate is hydrolyzed by a phospho-β-galactosidase to yield galactose 6-phosphate and glucose. Galactose 6-phosphate is catabolized through the tagatose 6-phosphate pathway. This pathway most likely exists in staphylococci exhibiting lactose PTS activity. In *S. xylosus* and probably other staphylococcal species that do not possess a lactose PTS, a permease is responsible for the transport of lactose. Galactose uptake has not been studied in these species. Nonphosphorylated lactose is hydrolyzed by a β-galactosidase to yield glucose and galactose. Galactose is likely catabolized through the Leloir pathway. In both staphylococcal species, glucose 6-phosphate, produced by a glucose kinase, enters the FBP pathway, the main glycolytic pathway in staphylococci. Only the encoded products of the galactoside-specific genes are shown. CM, cytoplasmic membrane; EII[lac], lactose-specific enzyme II; UDP-Gal, UDP-galactose; UDP-Glc, UDP-glucose; and Glc 6-P, glucose 6-P.

et al., 1978). The cloned lactose operon (Fig. 4) of *S. aureus* contains not only the phospho-β-galactosidase gene and genes involved in lactose uptake, but also the genes of the tagatose-6-P pathway (Breidt et al., 1987; Oskouian and Stewart, 1987). The corresponding genes are arranged in a heptacistronic operon (*lacABCD-FEG*). The locus was originally defined by mutations abolishing lactose utilization. The genes *lacFE* code for the galactoside-specific PTS permease, enzyme IIA, and enzyme IICB (Breidt and Stewart, 1986). The last gene, *lacG*, encodes the phospho-β-galactosidase. The *lacABCD* genes specify the enzymes of the tagatose-6-P pathway, with *lacAB* coding for galactose-6-P isomerase, *lacC* for tagatose-6-P kinase, and *lacD* for tagatose-1,6-diP aldolase (Rosey et al., 1991). The gene encoding the lactose operon repressor, *lacR*, is upstream of the *lacABCD-FEG* operon (Oskouian and Stewart, 1990a; Oskouian et al., 1990b). Utilization of lactose and galactose in *S. aureus* relies on the PTS-dependent uptake and phosphorylation of the sugars, resulting in lactose 6-P and galactose 6-P, respectively. Glucose and galactose 6-P are produced from intracellular lactose 6-P by a phospho-β-galactosidase (Hengstenberg et al., 1993). Glucose is metabolized via the FBP pathway, whereas galactose 6-P is degraded via the tagatose-6-P pathway.

Supplementation of the growth medium with lactose or galactose results in the induction of the *lac* genes, with galactose 6-P being the intracellular inducer. Apart from this sugar-specific regulation, the lactose operon of *S. aureus* is also subject to global carbon catabolite repression, but the nature of the regulatory mechanism remains unclear (Oskouian and Stewart, 1990a). It is conceivable that part of the described catabolite repression of the *lac* operon of *S. aureus* is actually caused by glucose-mediated inducer exclusion (Reizer et al., 1989; Saier et al., 1996).

The lactose metabolism pathway described for *S. aureus* is not universal for all staphylococci. In *S. xylosus*, for example, lactose is taken up in the nonphosphorylated form by a lactose permease, a member of the galactoside-pentoside-hexuronide (GPH) cation symporter protein family (Poolman et al., 1996). The lactose permease is encoded by *lacP* and the β-galactosidase by *lacH* (Bassias and Brückner, 1998). After lactose hydrolysis, glucose is phosphorylated by a glucose kinase and catabolized through the FBP pathway, and galactose is likely catabolized through the Leloir pathway (Fig. 3). The genes encoding the enzymes for the Leloir pathway from *S. carnosus* have recently been cloned (B. Krismer, unpublished data), and a gene encoding galactokinase has been isolated from an *S. xylosus* gene library (Brückner et al., 1993). The *lacR* gene of *S. xylosus*, upstream and in the opposite orientation of the *lacPH* operon, encodes an activator belonging to the AraC/XylS family. In contrast to *S. aureus*, *S. xylosus lacPH* operon is induced by only lactose, but not galactose, in the culture medium (Bassias and Brückner, 1998).

METABOLISM OF SUCROSE, MALTOSE, MANNITOL, RIBOSE AND XYLOSE   The sucrose PTS permease of *S. xylosus*, encoded by *scrA*, is composed of fused EIIBC domains (Wagner et al., 1993). The EIIA domain, which is essential for PTS-mediated sucrose uptake, has not been identified. On the basis of sucrose utilization in *E. coli* and *B. subtilis*, it appears questionable whether a separate sucrose-specific EIIA protein exists. In both of these organisms, the EIIA domain specific for glucose, either as an independent enzyme or fused to the IICB domains of the glucose permease, serves as phosphoryl donor for the sucrose-specific EII enzymes. The GlcA and GlcB proteins mentioned above would thus be good candidates for sucrose uptake factors.

Internalized sucrose 6-P is cleaved by sucrose-phosphate hydrolase, the gene product of *scrB*, to yield glucose 6-P and fructose (Brückner et al., 1993). Fructose is subsequently phosphorylated

Staphylococcus aureus lactose operon

Gene	Enzymes	AA
a) Lactose/galactose specific PTS transport		
lacF	lactose-specific IIA component	103
lacE	lactose-specific IIBC component	570
lacG	6-phospho-β-galactosidase	470
b) Tagatose 1,6-diphosphate pathway		
lacA	galactose-6-phosphate isomerase LacA subunit	142
lacB	galactose-6-phosphate isomerase LacB subunit	171
lacC	tagatose-6-phosphate kinase	310
lacD	tagatose 1,6-diphosphate aldolase	326
c) Repress or		
lacR	lactose phosphotransferase system repressor	251

Fig. 4. Lactose operon of *Staphylococcus aureus* (from Oskouian and Stewart [1990a] and Oskouian et al. [1990b]).

by a fructokinase encoded by *scrK* (R. Brückner, personal communication). The genes *scrB* and *scrK* are located next to each other and form an operon. The sucrose permease gene *scrA* is not located near the *scrBK* genes. Expression of *scrA* as well as *scrB* is induced by sucrose in the medium. Regulation is dependent on the LacI/GalR-type repressor ScrR, whose gene *scrR* is found upstream of *scrB* (Gering and Brückner, 1996). In addition to sucrose-specific ScrR-dependent regulation, the sucrose permease gene *scrA* is subject to carbon catabolite repression by the catabolite control protein CcpA (R. Brückner, personal communication).

An scr gene cluster, consisting of *scrR*, *scrB* and *scrK*, is also present in *S. aureus*. It is located next to *agrA*, the gene encoding the response regulator of the accessory gene regulator system (Agr; Novick et al., 1995). The gene for the sucrose permease has not yet been detected.

Maltose utilization in *S. xylosus* is dependent on an α-glucosidase (maltase), whose gene, *malA*, is found in the *malRA* operon; *malR* encodes a regulator belonging to the LacI/GalR family (Egeter and Brückner, 1995). The same enzymatic activity as mediated by MalA in *S. xylosus* has been characterized in *S. aureus*, and a gene with high sequence similarity has been identified in the *S. aureus* genome sequence. Therefore, both staphylococcal species probably use this enzyme to cleave maltose. However, the mechanism of maltose transport remains to be elucidated. In any case, at least one glucose moiety has to be phosphorylated by the glucose kinase for complete maltose catabolism.

The mannitol phosphotransferase system of *S. carnosus* has been described. The system consists of an EIICB enzyme (encoded by *mtlA*) and of EIIA (encoded by *mtlF*), which together form the mannitol-specific PTS permease. The *mtlA* and *mtlF* genes are clustered on the *S. carnosus* chromosome with *mtlD*, the gene for mannitol-1-phosphate dehydrogenase, which produces fructose 6-P. The gene *mtlA* is about 2 kb away from *mtlF* and *mtlD* (Fischer et al., 1989; Fischer and Hengstenberg, 1992). The nucleotide sequence of this intervening region has not been determined. *Staphylococcus aureus* possesses an EIIA protein specific for mannitol with the same apparent molecular mass as the *S. carnosu*s enzyme and virtually identical amino acid sequence at the amino terminus and around the phosphorylation site (Reiche et al., 1988). Thus, the *mtl* systems in the two organisms appear to be very similar.

The uptake of pentoses and pentitols is PTS-independent (Lehmer and Schleifer, 1980). Many staphylococcal species are unable to ferment pentoses, such as D-ribose, L-arabinose, and D-xylose, but are capable of pentose uptake.

For example, anaerobically grown *S. epidermidis* cells are unable to ferment ribose, mannitol, and lactose, but transport measurements confirm that ribose is taken up (Sivakanesan and Dawes, 1980). The genes encoding the enzymes for ribose utilization in *S. hyicus* have been cloned in *S. carnosus*, which thereby obtained the ability to utilize D-ribose (Keller et al., 1984). The *S. hyicus* DNA donor strain possessed D-ribokinase and D-ribose-5-P isomerase, which are absent in *S. carnosus*. Although the parent *S. carnosus* strain was unable to utilize ribose, the strain possessed a D-ribose-inducible uptake system that was severely repressed by the addition of glucose.

The xylose degradation genes of *S. xylosus* are organized in the operon *xylABR*, consisting of *xylR* (encoding the Xyl repressor), *xylA* (encoding xylose isomerase), and *xylB* (encoding xylulokinase; Sizemore et al., 1991). A xylose transporter is not part of the operon. The XylR protein acts as a repressor that binds to an operator site identified by footprint analysis (Sizemore et al., 1992). An interesting finding is that xylose is the inducer; gel-mobility shift experiments have shown that XylR is inactivated only in the presence of xylose, but not of xylose-P or other phosphorylated sugars. The *xyl* operon is also subject to glucose catabolite repression at the level of transcription; this control is independent of a functional *xylR* gene. Indeed, apart from the XylR operator sequence, a catabolite responsive element (*cre*) is also in front of *xylA*; *cre* is involved in glucose repression by binding activated CcpA (Hueck et al., 1994; Egeter and Brückner, 1996).

Catabolite Regulation

The major end product of anaerobic glucose metabolism in *S. aureus* is lactate (73–94%); acetate (4–7%) and traces of pyruvate are also formed (Theodore and Schade, 1965). During aerobic growth, acetate and CO_2 are the predominant end products; only 5–10% of the glucose carbon appears as lactate (Strasters and Winkler, 1963). In the presence of glucose, glycolysis is enhanced, and many enzymes of the pentose phosphate (PP) pathway and the tricarboxylic acid (TCA) cycle are suppressed; furthermore, the oxidation of pyruvate and the cytochrome content are decreased in glucose-grown *S. aureus* cells. Glucose-mediated catabolite repression is markedly pronounced in staphylococci.

The addition of glucose to aerobically grown *S. aureus* cells reduces both glucose degradation via the PP pathway and the subsequent oxidation of pyruvate via the TCA cycle. An investigation of the specific activity of various PP enzymes has

revealed no marked differences in extracts of *S. aureus* grown with or without glucose in nutrient broth. However, the specific activities of two enzymes of the FBP pathway (glyceraldehyde-3-P dehydrogenase and lactate dehydrogenase) are markedly increased in the presence of glucose. Furthermore, in the presence of glucose, the specific activities of the TCA cycle enzymes (succinate dehydrogenase and fumarase) are markedly decreased and fumarase activity is not detectable.

The reduced activities of the TCA cycle enzymes are very likely due to a repression of their biosynthesis; this phenomenon is referred to as the "glucose effect" or "carbon catabolite repression." The glucose-mediated inhibition of the TCA cycle activity could be triggered by the observed increase in the ATP pool. Citrate synthases of staphylococci are not affected by reduced nicotinamide adenine dinucleotide (NADH), but are severely inhibited by ATP (Hoo et al., 1971). In the presence of glucose, the nicotinamide adenine dinucleotide (NAD)-dependent L-lactate dehydrogenase of *S. aureus* has considerable activity during aerobic growth; although its activity is about tenfold less than that under anoxic conditions. It is therefore not surprising that in the presence of glucose, some lactic acid is produced even under oxic conditions (Garrard and Lascelles, 1968). During aerobic growth in the absence of glucose, an oxidation of acetate, succinate and malate by resting *S. aureus* cell suspensions has been observed manometrically (Collins and Lascelles, 1962; Strasters and Winkler, 1963). However, when glucose or galactose (0.04 M) is present in the growth medium, the oxidation of these substrates is abolished. Growth in the presence of glucose also results in a 40-fold decrease in the cytochrome content (Strasters and Winkler, 1963). The presence of a pyruvate dismutation system in staphylococci was first described by Krebs (1937). Pyruvate dehydrogenase activity has been demonstrated in *S. aureus* and *S. epidermidis* (Sivakanesan and Dawes, 1980).

REGULATION OF LACTATE DEHYDROGENASE ACTIVITY Anaerobically grown *S. epidermidis* cells ferment glucose with the production of lactate and trace amounts of acetate, formate, and CO_2. In *S. aureus*, glucose is metabolized principally via glycolysis and to a limited extent by the PP oxidative pathway (Sivakanesan and Dawes, 1980). However, certain staphylococcal species, such as *S. epidermidis* and *S. intermedius*, are distinguished by a fructose-1,6-bisphosphate (FBP)-activated, NAD-dependent L-lactate dehydrogenase (LDH; Götz and Schleifer, 1975; Götz and Schleifer, 1976). The enzyme has a total M_r of 130,000 and is composed of four subunits.

Physiological studies with *S. epidermidis* have shown that the intracellular concentration of FBP influences the LDH activity. During anaerobic growth and in the presence of glucose, a high intracellular concentration of FBP is reached, resulting in maximal LDH activity. In cells grown anaerobically in a glucose-limited medium, the FBP pool is exhausted because of glucose limitation. In this case, LDH is not fully saturated with FBP and its activity is not maximal. Similar results have been obtained with cells grown aerobically in a glucose-excess medium (Götz and Schleifer, 1978).

CLASS I ALDOLASE Aldolase is one of the key enzymes of the glycolytic pathway. There are two forms of fructose-1,6-bisphosphate aldolase which can be differentiated by their catalytic and structural properties. Class I aldolases function via the formation of a Schiff base intermediate between the substrate and a lysine amino group of the enzyme. Class II aldolases do not form a Schiff base intermediate, but contain an essential divalent cation, such as Zn^{+2}, Ca^{+2} or Fe^{+2}, and can be inhibited by EDTA. Since class I aldolases are typical for higher animals and plants and are only found in a few bacteria, it is surprising that nearly all staphylococcal species studied so far possess a class I aldolase (Götz et al., 1980). The only exceptions are *S. intermedius* and *S. hyicus*, which possess both classes of aldolases, and *S. caseolyticus* (reclassified as *Macrococcus caseolyticus*), which possesses only a class II aldolase (Fischer et al., 1982). The 33-kDa class I aldolase of *S. aureus* strain ATCC 12600 has been purified; it appears to be active as a monomer (Götz et al., 1980). Later, the *S. carnosus* TM300 *fda* gene, encoding the glycolytic fructose-1,6-bisphosphate aldolase (Fda), was cloned in *E. coli*. The 296-amino-acid protein has a M_r of 32,855. The *S. carnosus* FDA is also a class I aldolase (EC 4.1.2.13), which does not need divalent metal ions for catalytic activity. Cloning of *fda* back into *S. carnosus* led to a sixfold increase of aldolase production and activity. Glucose in the growth medium has a stimulating effect on aldolase production or activity (Witke and Götz, 1993).

CARBON CATABOLITE REPRESSION The availability of carbohydrates, especially of glucose, leads to regulatory processes often referred to as the "glucose effect" or "carbon catabolite repression" (Saier et al., 1995; Saier et al., 1996). Numerous publications on *S. aureus* describe the influence of glucose on a variety of cellular processes, such as utilization of alternative carbon sources, production of extracellular enzymes, activity of glycolytic enzymes, and

cytochrome content. Especially the production of potential virulence factors has attracted considerable attention. However, the mechanism by which glucose exerts its regulatory effect has not been elucidated. The analysis of catabolite repression of inducible systems is quite often complicated by the inability of the inducer to enter the cells in appreciable amounts, when glucose is also present in the medium. In addition to this process, referred to as "inducer exclusion/inducer expulsion," the rapid removal of internalized inducer, has been described for a number of Gram-positive bacteria (Saier et al., 1996). While inducer exclusion is found in a wide variety of bacteria, inducer expulsion is encountered less frequently. The latter does not appear to operate in *S. aureus*. The molecular mechanisms leading to inducer exclusion in Gram-positive bacteria are not yet completely understood.

In Gram-positive bacteria, one form of carbon catabolite repression relies on a transcriptional regulator, termed "catabolite control protein A" (CcpA; Henkin, 1996), a member of the GalR-LacI family of transcription factors (Weickert and Adhya, 1992). CcpA-dependent carbon catabolite repression has been analyzed in some detail in *S. xylosus* (Egeter and Brückner, 1996). Inactivation of the *ccpA* gene by insertion of a resistance cassette leads to a pleiotropic loss of transcriptional regulation at several promoters, including the *ccpA* promoters. This autoregulation of *ccpA* apparently reflects the need of *S. xylosus* to balance regulation by CcpA carefully. CcpA shows a relatively weak affinity for its cognate operator sites, termed "catabolite responsive elements" (*cre*; Hueck et al., 1994), and must, therefore, be activated to bind efficiently to *cre*. The signal enabling CcpA to bind to DNA is a phosphorylated form of HPr, the phosphocarrier protein of the PTS, which is produced by the HPr kinase (HPrK; Huynh et al., 2000). HPr is phosphorylated at Ser-46 in an ATP-dependent manner and can also be dephosphorylated by the same enzyme. Thus, HPrK constitutes a bifunctional HPr kinase/phosphatase. HPr as well as HPr kinase are absolutely required for CcpA activity in *S. xylosus* (Jankovic and Brückner, 2002). The genetic organization around the *hprK* gene of *S. xylosus* is identical to that in *S. epidermidis* and *S. aureus* (Huynh et al., 2000).

In addition to the loss of carbon catabolite repression, the *S. xylosus* HPr-kinase-deficient strain shows an unexpected phenotype. Its growth is inhibited by glucose. The mutant strain transports glucose at much higher rates than the wildtype strain, and it also produces methylglyoxal, which indicates unbalanced glucose metabolism. Therefore, HPr kinase is not only

important to trigger carbon catabolite repression, it is also needed to balance carbohydrate uptake and catabolic capacities of the cell. A summary of the current knowledge of CcpA-dependent catabolite repression in staphylococci and other Gram-positive bacteria with low DNA G+C content is shown in Fig. 5.

Gluconeogenesis

In the absence of a fermentable sugar source, heterotrophic microorganisms normally have to synthesize glucose or glucose 6-P. Pyruvate usually serves as a starting substrate. The pathway is essentially a reversal of glycolysis. Most of the glycolytic enzymes can also catalyze the reverse reaction. In gluconeogenesis, however, three bypass steps are involved: 1) pyruvate to phosphoenolpyruvate, 2) fructose 1,6-bisphosphate to fructose 6-P, and 3) glucose 6-P to glucose. The latter bypass is mostly found in mammals, where glucose has both signal and regulatory functions. Indeed, a glucose-6-phosphatase homolog has not yet been annotated and is very likely not necessary in staphylococci. Enzymes encoded by *pycA*, *pckA* and *fbp* represent the bypass enzymes, while the other enzymes belong to the FBP (fructose 1,6-bisphosphate) pathway and catalyze the reverse reaction.

The postulated gluconeogenesis pathway, according to the genes identified in the *S. aureus* N315 genome sequence (Kuroda et al., 2001), is shown in Fig. 6.

Oxidative Pentose Phosphate Cycle

The PP cycle is an essential cycle since it serves as the source of pentose phosphates, of which 5-phospho-ribosyl-1-pyrophosphate is required for the biosynthesis of nucleotides. According to the *S. aureus* N315 genome sequence, the relevant genes are present; only the lactonase gene has not yet been annotated. In Table 9, the corresponding annotated genes are listed.

Aerobic Respiration

TRICARBOXYLIC ACID CYCLE Not much is known about the biochemical properties or enzyme activities of the TCA cycle enzymes in staphylococci under various conditions. Most enzymatic activities were identified, e.g., aconitase (Somerville et al., 2002), isocitrate dehydrogenase, succinate dehydrogenase (Solozhenkin et al., 1991), fumarase, or malate dehydrogenase (Tynecka and Gajdzinska, 1967). In the genome sequence of *S. aureus* strains N315 and 8325, all the classical TCA cycle genes were annotated (Table 10). One therefore can assume that staphylococci
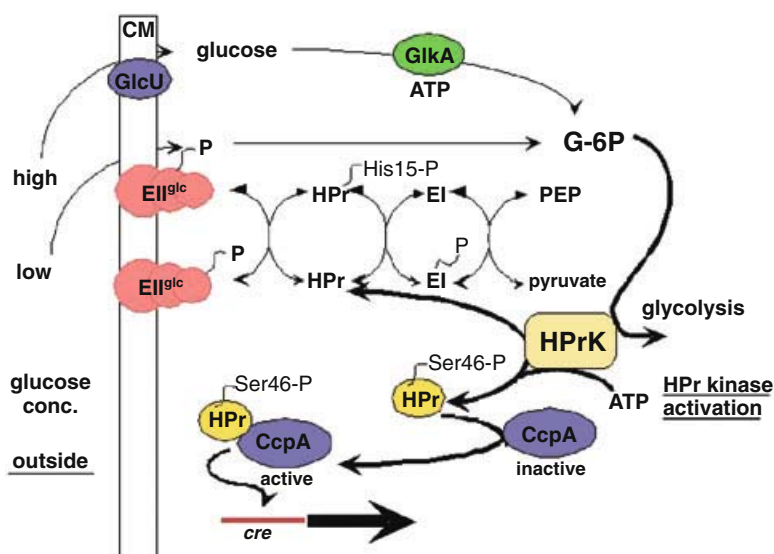
Fig. 5. Summary of the current knowledge on CcpA-dependent catabolite repression in staphylococci and other Gram-positive bacteria with low G+C content. When provided with a rapidly metabolizable phosphotransferase system (PTS) sugar (e.g., glucose), bacteria transport it via the PTS. During the process of sugar transport, the phosphoryl group is transferred to the entering sugar, and the PTS proteins are predominantly left in a non-phosphorylated form. Heat-stable phosphocarrier (HPr) kinase senses the presence of glucose in the cell, most probably via intermediates of glycolysis, and phosphorylates non-phosphorylated HPr at Ser-46 in an ATP-dependent manner. By interacting with the central regulator CcpA, P-Ser-46-HPr increases its affinity for cre operators. The complex CcpA/HPr-Ser46-P/*cre* blocks or activates the transcription of different operons. In the absence of glucose, the activity of HPrK shifts towards P-Ser-HPr phosphatase. Since there is no available sugar and thus no phosphoryl group transfer by the PTS, the HPr protein remains phosphorylated at His-15. Without P-Ser-46-HPr as a co-effector, CcpA shows very low affinity for binding to *cre* elements, and as a consequence, CcpA does not regulate the catabolic operons. It is assumed that in the presence of high glucose concentrations, glucose is mainly transported independently from the PTS via the permease GlcU in a non-phosphorylated form and is subsequently phosphorylated by glucose kinase, GlkA.

Table 9. Genes of the oxidative pentose phosphate cycle in *S. aureus* N315 and *S. aureus* NCTC 8325.

Gene designation	Encoded enzyme
—	Glucose-6-phosphate dehydrogenase
gnd	Phosphogluconate dehydrogenase (decarboxylating)
rpi	Similar to ribose-5-phosphate isomerase
cfxE	Ribulose-5-phosphate 3-epimerase homolog
—	Similar to transaldolase
tkt	Transketolase

Symbol: –, not yet assigned.
[a]Genome sequenced by Kuroda et al. (2001).
[b]Sequenced at the University of Oklahoma Health Sciences Center.

Table 10. Tricarboxylic acid cycle genes.

Gene designation	Gene function
citZ	Citrate synthase II
citB	Aconitate hydratase
citC	Isocitrate dehydrogenase
odhA	2-Oxoglutarate dehydrogenase E1
odhB	Dihydrolipoamide succinyltransferase
sucC	Succinyl-CoA synthetase (β-subunit)
sucD	Succinyl-CoA synthetase (α-subunit)
sdhC	Succinate dehydrogenase cytochrome b-558
sdhA	Succinate dehydrogenase flavoprotein subunit
sdhB	Succinate dehydrogenase iron-sulfur protein subunit
citG	Fumarate hydratase, class-II
mdh	Malate dehydrogenase homolog

From Kuroda et al. (2001).

have in principle a complete TCA cycle; however, bottlenecks exist.

The TCA cycle is fuelled by acetyl CoA and the responsible enzyme is the pyruvate dehydrogenase (PDH), which catalyzes the oxidative decarboxylation of pyruvate, to form acetyl-CoA. The *S. aureus* PDH complex has been isolated and the last gene of the PDH operon, *pdh*D, which encodes the lipoamide dehydrogenase (LPD) has been sequenced (Hemila, 1991).

The *S. aureus* genome sequence revealed the E_2 core complex subunit and the peripheral subunits E_1 and E_3 (Table 11). The *S. aureus* genome contains also a typical pyruvate carboxylase gene that is necessary for initiation of the TCA cycle (Table 6). The activity of this enzyme has been verified.

Pyruvate

ATP ⌐→ + HCO₃⁻   **Pyruvate**
ADP ←┘   **carboxylase (pycA)**

Oxaloacetate
A(G)TP ⌐→   **PEP-**
A(G)DP ←┘   **carboxykinase (pckA)**
PEP

↕   **Enolase**
                         Mutase

3-P-glycerate
ATP ⌐→   **P-glycerate**
ADP ←┘   **kinase**

1,3-P-glycerate
   ⌐→ NADH   **GAP-DH**
   └→ NAD+   **TPI**
                         Aldolase

Fructose-1,6-P
ATP ⌐→   **Fructose-**
ADP ←┘   **bisphosphatase**

Fructose-6-P
                         Isomerase
↕

Glucose-6-P

⋮
↓

(Glucose ?)
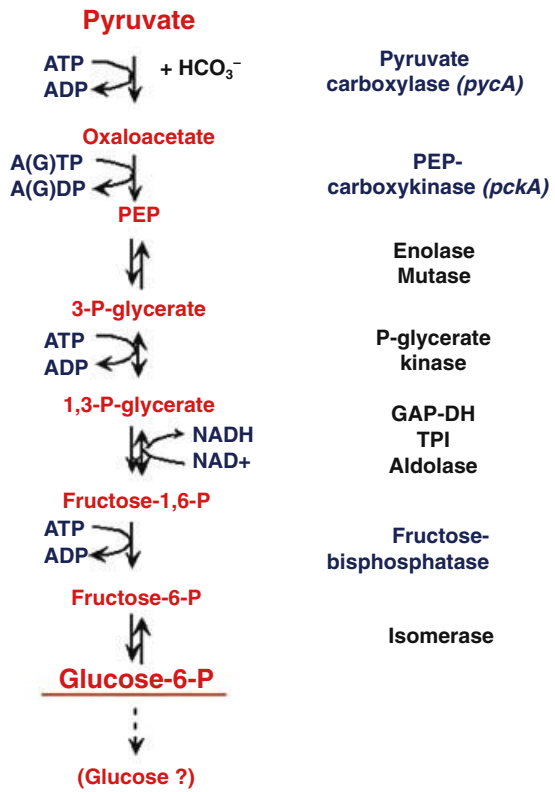
Fig. 6. Proposed gluconeogenesis pathway in *S. aureus*. PEP, phosphoenol pyruvate; GAP-DH, D-glyceraldehyde-3-phosphate dehydrogenase; and TPI, triose phosphate isomerase.

Table 11. Entrance into the TCA-cycle.

Gene designation	Gene function
1. Pyruvate-DH (E1, E2 and E3 components)	
Pyruvate → acetyl-CoA + CO₂	
phdB	Pyruvate dehydrogenase E1 component β-subunit
pdhC	Dihydrolipoamide S-acetyltransferase E2 component[a]
	Acetyl-CoA + dihydrolipoamide ↔ CoA + S-acetyldihydrolipoamide
pdhD	Dihydrolipoamide dehydrogenase component E3
2. Pyruvate carboxylase	
Pyruvate + HCO₃⁻ + ATP ↔ Oxalacetate + ADP + Pi	
pycA	Pyruvate carboxylase

[a]Hemila (1991).
From Kuroda et al. (2001).

RESPIRATORY CHAIN Taber and Morrison (1964) identified three cytochromes in *S. aureus*: cytochrome *a*-602 and cytochromes *b*-555 and *b*-557. The two *b*-type cytochromes have different reactivities. Cytochrome *b*-557 is reduced in the presence of 2-heptyl-4-hydroxyquinoline-*N*-oxide

(HOQNO), while all the other hemoproteins of the respiratory chain remain oxidized. In contrast to cytochromes *a*-602 and *b*-555, cytochrome *b*-557 is not reduced by ascorbate-dichlorophenol-indophenol, suggesting that it has a lower reduction potential than the other cytochromes. The most significant difference, however, is that cytochrome *b*-557, unlike cytochromes *b*-555 and *a*-602, does not react with carbon monoxide. Cytochrome *b*-557 appears to be an intermediate electron carrier of the cytochrome *b* or *b₁*-type. Cytochrome *b*-555, which is also found in *S. epidermidis*, binds carbon monoxide and probably is the major terminal oxidase; it is therefore, now referred to as "cytochrome *O*." There are some indications that the prosthetic groups of cytochromes *O* and *b*-557 are protohemins. The role of cytochrome *a*-602 as a terminal oxidase is less clear. It may be involved with nitrate reductase activity. In particulate enzyme preparations from *S. aureus*, succinate oxidase, NAD-linked ethanol oxidase, and NADH-oxidase activities are detectable. Succinate oxidase activity is inhibited by ultraviolet (UV) light (340 nm) and surprisingly by amytal (barbiturate A), which inhibits electron transfer from NADH to ubiquinone in higher organisms. The two oxidation-reduction dyes, methylene blue and 2,6-dichlorophenol-indophenol interact with the electron transfer system. The reduced form of 2,6-dichlorophenol-indophenol reduces cytochrome *a*-602 and cytochrome *O* without reducing cytochrome *b*-557. It was found that trimethylamine-*N*-oxide (TMAO) inhibited the growth of *S. aureus* but not of *S. epidermidis*. This selective inhibition of *S. aureus* was based on the inhibition of the electron transport system by oxidizing the cytochromes in *S. aureus*. It was suggested that the inhibition occurred between cytochrome *b* and cytochrome *O* (Suzuki et al., 1992).

Analysis of cytochrome spectra of *S. aureus* and other staphylococcal species revealed two more minor cytochromes of the *b*-type in addition to cytochromes *a*-602, *b*-557, and *O*-555 (Faller et al., 1980). Cytochrome *b*-552 is found in all staphylococcal species except *S. sciuri*. Cytochromes *b*-560 and *b*-566 are widely distributed in staphylococci. Cytochromes of the *c*-type (e.g., *c*-549 and *c*-554) have been only found so far in *S. sciuri*, *S. lentus* and *Macrococcus caseolyticus*.

On the basis of the presence of cytochrome *c*, modified oxidase and benzidine tests have been developed which allow rapid differentiation between staphylococci on one hand and micrococci and macrococci on the other hand (Faller and Schleifer, 1981).

Staphylococci possess menaquinones (MK, vitamin K₂) as their sole isoprenoid quinones.

There is a species-specific variation with regard to the length of the isoprenoid side chains. The two principal menaquinones have seven to eight isoprene units (Collins and Jones, 1981). Menaquinones are located in the cytoplasmic membrane (White and Frerman, 1967) and play important roles in electron transport and oxidative phosphorylation.

Staphylococcus aureus uses aerobically very likely menaquinone oxidase(s) and at least four quinol oxidase-like genes (*qox*A, B, C and D) were identified. Since the *S. aureus qoxA* to *qoxD* genes are very similar to the corresponding *B. subtilis* genes, we expect these genes will have a similar function. As all analyzed staphylococcal species possess a cytochrome *a*-605, we believe that this cytochrome is part of the *S. aureus* Qox system and functions as a second terminal oxidase. Very likely the ubiquitous cytochrome *b*-552 (Faller et al., 1980) is associated with cytochrome *a*-605.

Moreover, the *S. aureus* genome sequences revealed two putative cytochrome *d* menaquinol oxidases: subunit I homolog (*cydA*) and subunit II homolog (*cydB*). Since a *d*-type cytochrome has never been observed in the staphylococcal cytochrome spectrum (Faller et al., 1980), it is possible that CydAB complex represents the cytochrome *O*-555 menaquinol oxidase. The very few bits of information on the respiratory components of staphylococci suggest that they have a branched respiratory system consisting of two alternative and menaquinol-dependent terminal oxidases, a cytochrome *bo* and a cytochrome *aa3* oxidase. The still limited knowledge on the overall electron transfer sequence is illustrated in Fig. 7. The scheme is necessarily incomplete since the components of the respiratory chains of *S. aureus* and other staphylococci are not yet fully identified.

The *S. aureus* genome sequence revealed the presence of a typical F_0F_1-ATP synthase [EC:3.6.3.14] with the following gene order of the various subunits: epsilon (*atpC*), beta (*atpD*), gamma (*atpG*), alpha (*atpA*), delta (*atpH*), b (*atpF*), c (*atpE*), and a (*atpB*) subunits.

Anaerobic Respiration

In respiration, nitrate is used as an alternative electron acceptor when oxygen is not available. The enzymes for this pathway are only found in bacteria. Two main forms have been described so far; in both, nitrate reduction is coupled to the generation of a proton motive force (p; Boonstra and Konings, 1977; Jones et al., 1980), which is directly utilized as a source of energy or transformed to ATP by a membrane-associated ATPase.

Despite its broad application in food technology, little was known about the biochemistry and genetics of nitrate reduction in *S. carnosus*. A physiological characterization of nitrate and nitrite reduction in *S. carnosus* (Neubauer and Götz, 1996) revealed the presence of a typical dissimilatory nitrate reductase with the following features: 1) repression and inhibition by oxygen, 2) induction by anaerobiosis and nitrate or nitrite, 3) no inhibition by ammonia, 4) energy gain under anaerobic conditions, and 5) localization in the membrane.

Nitrite reductase in *S. carnosus* is a NADH-dependent cytosolic enzyme that forms ammonia as the only end product. The enzyme is regulated similar to dissimilatory enzymes, i.e., it is 1) repressed and inhibited by oxygen, 2) induced by anaerobiosis and nitrate or nitrite, and 3) not inhibited by its end product ammonia. The relatively low energy gain with nitrite under anaerobic conditions points towards a function as terminal electron acceptor, which might be important for the anaerobic recycling of NADH (Neubauer and Götz, 1996). In cells of *S. carnosus*, nitrate is reduced to ammonia in two steps. The first step includes uptake of nitrate, its reduction to nitrite and the subsequent excretion of nitrite. The second step takes place after depletion of nitrate: the accumulated nitrite is imported and reduced to ammonia, which accumulates in the medium. In growth yield studies, nitrate reduction appeared energetically more favorable than nitrite reduction, which makes sense since nitrate reduction is most likely coupled to the generation of a proton motive force. A comparison of the turnover rates in whole cells with glucose as electron donor reveals that nitrate reduction is 10-fold faster than nitrite reduction. For the inhibition of nitrite reduction by nitrate, two possible mechanisms are discussed which are not mutually exclusive. Both are based on the higher activity of nitrate compared to nitrite reductase. Both enzymes compete for NADH, which is in vivo the most important electron
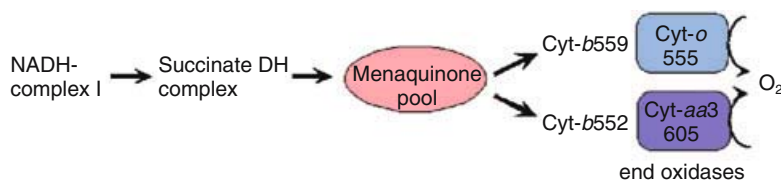
Fig. 7. Proposed respiratory system in staphylococci.

donor. Owing to its higher specific activity, nitrate reductase is expected to perform the bulk of NADH oxidation. In addition, the high rate of nitrate reduction could lead to an internal accumulation of nitrite, which could be the result of a less efficient nitrite reduction or export. High concentrations of nitrite (100 mM) effectively inhibit nitrite reductase but not nitrate reductase activity (Neubauer and Götz, 1996).

THE NITRATE REDUCTASE OPERON (NARGHJI) The enzyme that catalyzes the energy-gaining reduction of nitrate to nitrite is well characterized in *E. coli*. The membrane-bound dissimilatory nitrate reductase (NRA) is an enzyme complex composed of three subunits ($a_2b_2g_4$). The corresponding genes are organized in an operon, *narGHJI* and *narZYWV*. The NarJ protein is believed to be involved in the assembly of the native enzyme complex (Dubourdieu and DeMoss, 1992). The γ-subunit (NarV), a *b*-type cytochrome, receives electrons from the quinone pool. The electrons are then transferred via the iron-sulfur clusters of the β-subunit (NarY) to the molybdenum cofactor bound to the α-

subunit (NarG and NarZ, respectively; Blasco et al., 1989).

To gain more insight into the genetic basis of dissimilatory nitrate reduction, a transposon Tn*917* mutagenesis in *S. carnosus* TM300 was performed and mutants defective in the conversion of nitrate to nitrite were selected and analyzed. Various nitrate-reductase-negative mutants led to the identification of a similar nitrate reductase operon, *narGHJI*, as described for *E. coli* (Pantel et al., 1998). Unlike in *E. coli*, in *S. carnosus* there is no evidence for a second operon. The organization of genes and operons involved in dissimilatory nitrate/nitrite reduction are shown in Fig. 8.

Transcription from the *nar* promoter is induced by anaerobiosis, nitrate and nitrite. This is in accordance with the nitrate reductase activities determined with benzyl viologen as electron donor. However, in the presence of oxygen and nitrate, high transcription initiation but low nitrate reductase activity was observed. Since the nitrate reductase formed during anaerobic growth was insensitive to oxygen, other oxygen-sensitive steps (e.g., postranscriptional mechanisms and molybdenum cofactor biosynthesis)

A) Genetic organization of the nitrite/nitrate gene locus

nirC, nitrite transporter
nir/sir, nitrite reductase operon
nar, nitrate reductase operon
oxygen sensor, nitrate regulator
narT, nitrate transporter
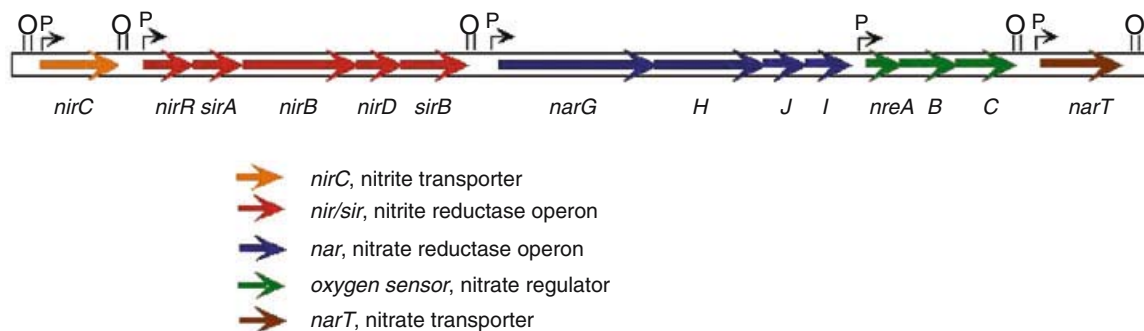
B) Genetic organization of the molybdo-cofactor biosynthesis genes

mod, molybdate transport
moa and moa genes most likely involved in biosynthesis of molybdopterin
mob genes most likely involved in insertion of GMP
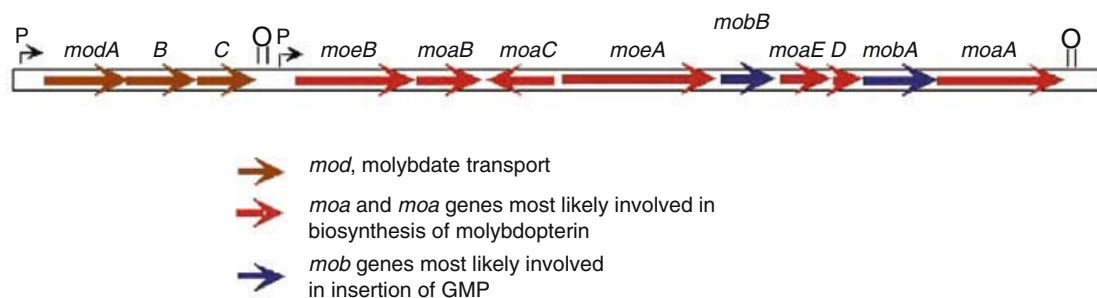
Fig. 8. Genetic organization of genes and operons involved in the dissimilatory nitrate/nitrite reduction in *Staphylococcus carnosus*.

must be involved. In *E. coli*, the *nar* promoter is induced by the *trans*-acting factors ferredoxin-NADP reductase (Fnr) and phosphorylated NarL; Fnr is activated in response to oxygen deprivation and NarL is the response regulator of a two-component system that signals the presence of nitrate. In the *S. carnosus nar* promoter, no conspicuous Fnr and integration host factor (IHF) recognition sites are present; there is only one site that is related to the *E. coli* NarL consensus sequence.

THE NITRITE-REDUCING SYSTEM Characterization of a nitrite-reductase-negative *S. carnosus* Tn*917* mutants led to the identification of the *nir* operon, which is comprised of five genes: *nirR*, *sirA*, *nirB*, *nirD* and *sirB* (Neubauer et al., 1999a). The NirBD proteins represent the dissimilatory NADH-dependent nitrite reductase, SirA and SirB (both necessary for biosynthesis of the siroheme prosthetic group), the putative oxidase/chelatase and the uroporphyrinogen III methylase, respectively, and NirR, a protein with known function. It was suggested that NirR is essential for *nir* promoter activity (Neubauer et al., 1999a). In the absence of NirR, a weak promoter upstream of *sirA* seems to drive transcription of *sirA*, *nirB*, *nirD* and *sirB* in the stationary growth phase. Nitrite reduction in *S. carnosus* does not occur in the presence of oxygen and nitrate (Neubauer and Götz, 1996), which is in agreement with the fact that anaerobiosis, nitrite, and nitrate induce the *nir* promoter. Although a transcript is detectable, no nitrite reduction occurs in cells grown aerobically with nitrate or nitrite, indicating an additional oxygen-controlled step at the level of translation, enzyme folding, assembly, or insertion of prosthetic groups. The nitrite-reducing activity expressed during anaerobiosis is switched off reversibly when the oxygen tension increases, most likely owing to the competition for electrons with the aerobic respiratory chain. Another gene, *nirC*, is located upstream of the *nir* operon and encodes a putative integral membrane-spanning protein with unknown function. A *nirC* mutant showed no distinct phenotype. In *S. carnosus*, nitrate is first reduced to nitrite evidently by a dissimilatory enzyme. Nitrite accumulates in the growth medium and is further reduced by a dissimilatory NADH-dependent nitrite reductase. Nitrite reduction only occurs in the absence of nitrate.

The *narT* mutant displayed wildtype levels of nitrate reductase activity but, unlike the wildtype strain, does not take up nitrate and accumulate nitrite when grown in the presence of nitrate under anaerobic conditions (Fast et al., 1997). The 41-kDa NarT is a highly hydrophobic trans-

membrane protein of 388 amino acids. Its protein sequence shows similarity to *B. subtilis* NasA (25.8% identity) and *E. coli* NarK (22.8% identity).

MOLYBDOPTERIN COFACTOR BIOSYNTHESIS All molybdoenzymes except nitrogenase contain a unique form of molybdopterin as cofactor, sometimes conjugated to a nucleoside monophosphate (Rajagopalan and Johnson, 1992). At least five different loci in *E. coli* are involved in molybdate transport and processing, synthesis of molybdopterin, and conversion to the dinucleotide form (Rajagopalan, 1996).

In *S. carnosus*, nine genes were identified (Fig. 8), all of which appear to be involved in molybdenum cofactor biosynthesis (Neubauer et al., 1998). The regulation and function of one of the proteins, MoeB, was further analyzed. In addition, *moeB* Tn*917*-insertion mutants showed no nitrate reductase activity because they were molybdenum-cofactor-deficient. However, nitrate reductase activity in cell-free extracts could be reconstituted with a low-molecular-weight component (most likely free molybdenum cofactor) from an *S. carnosus* mutant that is defective in the nitrate reductase structural genes. Expression studies of *moeB* indicated that anaerobiosis and nitrate each enhance transcription of *moeB* (Neubauer et al., 1998).

MOLYBDATE TRANSPORT SYSTEM MODABC In *S. carnosus* three genes, *modABC*, were identified which encode an ATP-binding cassette (ABC) transporter that is involved in molybdate transport (Neubauer et al., 1999b). These genes are located directly upstream of *moeB* and seem to be part of the molybdenum cofactor biosynthesis gene cluster. The *mod* mutants are devoid of nitrate reductase activity. It was shown by [^{14}C]-palmitate labeling that ModA represents a lipoprotein that, in Gram-positive bacteria, is the counterpart of the periplasmic binding proteins of Gram-negative organisms. The sequence characteristics identify ModB as the integral-membrane, channel-forming protein and ModC as the ATP-binding energizer for the transport system. Mutants defective in *modABC* revealed only 0.4% of wildtype nitrate reductase activity. Molybdate at a nonphysiologically high concentration (100 μM) fully restored nitrate reductase activity, suggesting that at least one other system is able to transport molybdate but with lower affinity. The expression of *modA* (and most likely *modBC*) was independent from oxygen and nitrate. To date, there are no indications for molybdate-specific regulation of *modABC* expression since in a *modB* mutant, *modA*

expression was unchanged and no different from that in the wildtype strain (Neubauer et al., 1999b).

Recently in *S. carnosus*, the *nreABC* (for nitrogen regulation) genes were identified and shown to link the nitrate reductase operon (*narGHJI*) and the putative nitrate transporter gene *narT*. An *nreABC* deletion mutant was dramatically affected in nitrate and nitrite reduction and growth. The data provide evidence for a global regulatory system important for aerobic and anaerobic metabolism, with NreB and NreC forming a classical two-component system and NreB acting as a sensor protein with oxygen as the effector molecule (Fedtke et al., 2002).

Homologous sequences of the described nitrate/nitrite reductase systems in *S. carnosus* are also identifiable in the *S. aureus* N315 and *S. epidermidis* RP62A genomes. Thus the ability to use nitrate as an alternative terminal electron donor appears to be widely distributed among staphylococci.

Cell Wall Peptidoglycan

Staphylococcus aureus was one of the first bacterial species where peptidoglycan biosynthesis was studied and pioneering results achieved. A comprehensive description is presented in the monographs by Rogers et al. (1980b) and Ghuysen and Hakenbeck (1994). The staphylococcal peptidoglycan is a heteropolymer consisting of glycan strands crosslinked by peptides. According to the classification by Schleifer and Kandler (1972), *S. aureus* has an A3α peptidoglycan type. The peptide chain length is generally long in Gram-positive bacteria, yet the most extensively crosslinked peptidoglycan, that of *S. aureus*, has an average of about 15 repeating units and a maximal chain length of 30–40 peptide units (Snowden and Perkins, 1990; Henze et al., 1993). In exponentially grown cells in rich medium, more than 95% of the subunits are crosslinked in *S. aureus*. This extremely high degree of crosslinking is possible only because their long and flexible pentaglycine interpeptide bridges are able to span distances between peptides otherwise much too far apart to be crosslinked (Ghuysen and Hakenbeck, 1994).

The peptidoglycan of staphylococci is also exceptional in that, apart from the small proportion of residual uncross-linked primary pentapeptide side-chains, it has almost no free carboxyl groups, since the D-glutamic acid α-carboxyl group is amidated (Tipper et al., 1967; Schleifer, 1975a; Pucci et al., 1995).

Staphylococcus aureus also provides an example of the second type of peptidoglycan modification, the presence of *O*-acetyl substituents on the *N*-acetylmuramic acid residues. In this organism about 50% of the muramic acid residues are present as the 4-*N*, 6-*O*-diacetylderivative (Tipper et al., 1971). This substitution has the effect of making the peptidoglycan resistant to the muramidase egg-white lysozyme (Warren and Gray, 1965).

Additions to the peptidoglycan of *S. aureus* also occur in the form of covalently linked wall teichoic acid. They are attached by phosphodiester linkages on C_6 of some of the muramic acid residues (Hay et al., 1965). In *S. aureus*, approximately 7.7% of the muramic acid is phosphorylated. Suggesting that on approximately every thirteenth muramic acid a wall teichoic acid is covalently linked.

As in all staphylococci, consecutive cell divisions are initiated at an angle of 90 degrees in three dimensions, sometimes even before completion of the cell separation process of the first division plane. Cell division is achieved by the formation of a highly organized cross wall, which is initiated asymmetrically and eventually fuses in the center of the cell to form a complete cross wall. On the basis of distinct morphological features, as revealed by electron microscopy, involvement of several autolytic systems has been suggested in cell division process. One of these, the splitting system, appears as a ring of periodically arranged tubules in the center of the cross wall (Giesbrecht et al., 1998).

Teichoic acid-like material has been chemically associated with the splitting system (Morioka et al., 1987). During isolation and purification of the peptidoglycan, the morphological appearance of the splitting system remains detectable until the final purification step, i.e., removal of the teichoic acid. If the splitting system can no longer be detected, two separated pieces of cross wall are visible. Once the cross wall has been completed, cell separation is initiated by highly organized entities called "murosomes," which punch tiny holes into the peripheral wall along the division plane (Giesbrecht et al., 1998).

BIOSYNTHESIS OF PEPTIDOGLYCAN The overall peptidoglycan synthesis can be divided into three distinct stages (Rogers et al., 1980b): 1) The formation of the nucleotide sugar-linked precursors, UDP-*N*-acetylglucosamine, UDP-*N*-acetylmuramic acid and UDP-*N*-acetylmuramyl-pentapeptide; 2) the transfer of phospho-*N*-acetylmuramyl-pentapeptide and *N*-acetylglucosamine to the lipophilic carrier, undecaprenyl phosphate, to yield a disaccharide-(pentapeptide)-pyrophosphate-undecaprenol; and 3) the transfer of this complete subunit to the growing peptidoglycan. At this stage, cross-bridge formation occurs together with secondary modification of the newly synthesized peptidoglycan. Known genes

(enzymes) involved in the pathway of peptidoglycan biosynthesis are shown in Fig. 9, and in Table 12, annotated peptidoglycan biosynthesis genes of *S. aureus* N315 are listed.

UDP-*N*-acetylmuramic acid is formed from UDP-*N*-acetylglucosamine (UDP-Glc*N*ac), UTP and *N*-acetylglucosamine-1-P. The synthesis of UDP-Glc*N*ac starts from fructose-6-P and involves UTP-glucose-1-P uridyltransferase (GtaB), glucosamine-fructose-6-P aminotransferase (GlmS), and phosphoglucosamine-mutase GlmM (FemD). The UDP-*N*-acetylglucosamine-pyrophosphorylase reaction is also involved in the synthesis of many other nucleotide-linked sugars. The next step is the formation of the first intermediate UDP-*N*-acetylmuramic acid. These reactions involve the transfer of a pyruvate enol ether from phosphoenolpyruvate to UDP-*N*-acetylglucosamine and its subsequent reduction to yield UDP-*N*-acetylmuramic acid (Wickus et al., 1973). The enzyme involved is UDP-*N*-acetylglucosamine 1-carboxyvinyl transferase. In the genome sequence, there are two transferases present, MurA and MurZ (MurB). The transferase activity was initially detected in cell-free

BIOSYNTHESIS OF PEPTIDOGLYCAN
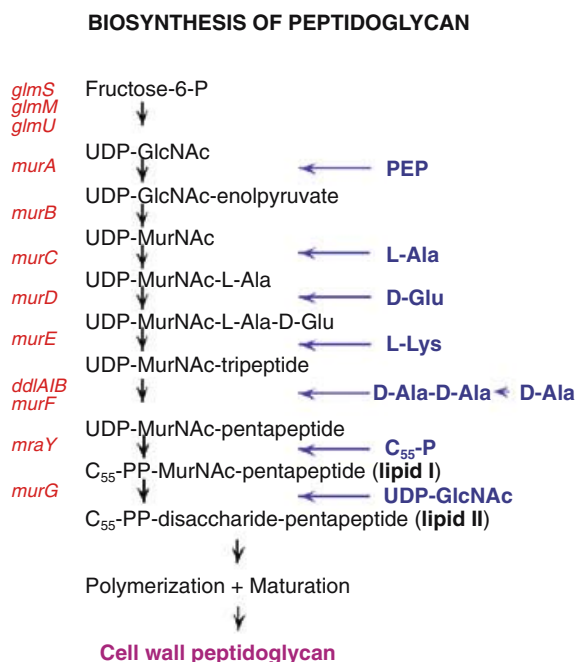
Fig. 9. Pathway of peptidoglycan biosynthesis in staphylococci.

Table 12. Annotated peptidoglycan biosynthesis genes of *S. aureus* N315.

Gene designation	Encoded enzyme
glmS	Glucosamine-fructose-6-phosphate aminotransferase
glmM (*femD*)	Phosphoglucosamine-mutase
gtaB	UTP-glucose-1-phosphate uridyltransferase
murA	UDP-*N*-acetylglucosamine 1-carboxyvinyl transferase 1
murZ	UDP-*N*-acetylglucosamine 1-carboxylvinyl transferase 2
murC	UDP-*N*-acetylmuramate-alanine ligase
ddlA	D-Alanine-D-alanine ligase
murF	UDP-*N*-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate-D-alanyl-D-alanyl ligase
murI	Glutamate racemase
murD	UDP-*N*-acetylmuramoylalanine-D-glutamate ligase
mraY	Phospho-*N*-muramic acid-pentapeptide translocase
murG	Undecaprenyl-PP-Mur*N*Ac-pentapeptide-UDPGlc*N*Ac Glc*N*Ac transferase
murE	UDP-*N*-acetylmuramoylalanyl-D-glutamate-2,6-diaminopimelate ligase
sgtA	Probable transglycosylase
sgtB	Hypothetical protein, similar to penicillin-binding protein 1A/1B
uppS	Undecaprenyl pyrophosphatase synthetase
pbp4	Penicillin binding protein 4
bacA	Bacitracin resistance protein (putative undecaprenol kinase) homologue
femA	Factor essential for expression of methicillin resistance
femB	FemB protein
uppS	Undecaprenyl pyrophosphatase synthetase
pbpA	Penicillin-binding protein 1
pbp2	Penicillin-binding protein 2
pbp3	Penicillin-binding protein 3
sgtB	Hypothetical protein, similar to penicillin-binding protein 1A/1B
NN	Similar to UDP-*N*-acetylglucosamine pyrophosphorylase
mraY	Phospho-*N*-muramic acid-pentapeptide translocase
fmhA	FmhA protein
fmhB	FmhB protein
fmhC (*eprh*)	FmhC protein
srtA	Sortase
drp35	Drp35
ebsB	Cell wall enzyme

From Kuroda et al. (2001).

extracts from *S. aureus* (Strominger, 1965). It was partially purified from *S. epidermidis* (Wickus et al., 1973). The reaction is reversible and moreover specifically and irreversibly inhibited by fosfomycin. The UDP-Glc*N*Ac-enolpyruvate reductase was also purified from *S. aureus* (Wickus et al., 1973). In all cases, NADPH is used as co-factor. The synthetases that catalyze the assembly of the peptide moiety of the peptidoglycan unit are cytoplasmic.

The UDP-Mur*N*Ac pentapeptide is formed by the sequential addition of L-alanine, D-glutamic acid, L-lysine and D-alanyl-D-alanine to the D-lactyl group of UDP-Mur*N*Ac. Each step is catalyzed by a specific synthetase using ATP. The involved enzymes are MurC, MurD, MurE and DdlA (see Table 9). The end product is UDP-Mur*N*Ac-L-Ala-D-*iso*-Glu-L-Lys-D-Ala-D-Ala (also named "UDP-Mur*N*Ac-pentapeptide" and "Park's nucleotide").

The conversion of L-Glu to D-Glu is catalyzed by MurI. The conversion of L-Ala to D-Ala is catalyzed by alanine-racemase, an enzyme studied in detail in *S. aureus* (Cheung et al., 1983; Kullik et al., 1998b). D-Alanyl-D-alanine is formed by the dimerization of D-alanine. The enzyme involved, D-alanine:D-alanine ligase, has been already purified from *S. aureus* by Ito and Strominger (1973). The enzyme requires a "heat-stable co-factor," Mg^{+2} (or Mn^{+2}), K^+ and ATP for activity (for a review, see Rogers et al., 1980a).

Under certain conditions, glycine has been shown to replace both alanine isomers in UDP-*N*-acetylmuramyl-pentapeptide. Growth of *S. aureus* in the presence of high concentrations of glycine resulted in the accumulation of modified UDP-*N*-acetylmuramyl-pentapeptides containing both glycyl-D-alanine and D-alanyl-glycine as the terminal dipeptide (Hammes et al., 1973). Thus, under these rather special conditions the substrate specificities of both the D-alanine ligase and the UDP-*N*-acetylmuramyl-3-peptide:D-Ala-D-Ala-ligase have been overridden. The synthesis and subsequent incorporation into peptidoglycan of such natural precursors may contribute to the inhibitory effects of glycine and D-amino acids on many bacteria.

BIOSYNTHESIS OF THE LIPID II PRECURSORS Undecaprenol, originally present either as the free alcohol or as a phosphorylated derivative has been isolated in pure form from *S. aureus* (Higashi et al., 1970). The 17-kDa phosphokinase of *S. aureus* is soluble in several organic solvents but insoluble in water and has an unusually high content (58%) of nonpolar amino acids. Subsequent purification allows the separation of an apoprotein to which activity can be restored

by a wide range of natural and synthetic phospholipids and detergents. Initially it was suggested that phosphatidylglycerol or diphosphatidylglycerol (cardiolipin) were specific lipid co-factors. However, enzymatic activity appears to be more dependent on "lipid hydration" rather than lipid viscosity or the actual chemical structure of the polar group of the activating lipid (Sanderman, 1976). In stationary-phase cultures, free undecaprenol was present in large excess, representing approximately 80% of the total peptidoglycan lipid.

UDP-Mur*N*Ac-pentapeptide is phosphodiester linked to an undecaprenyl-pyrophosphate carrier molecule at the expense of UDP to yield C_{55}–PP-Mur*N*Ac-L-Ala-D-*i*Gln-L-Lys-D-Ala-D-Ala, or lipid I (Higashi et al., 1970). UDP-Glc*N*Ac is linked to the muramoyl moiety to generate the disaccharide lipid II precursor [C_{55}-PP-Mur*N*Ac(-L-Ala–D-*i*Gln–L-Lys(Gly5)-D-Ala-D-Ala)-β1-4-Glc*N*Ac]. Lipid I and lipid II precursors are hooked at the inner side of the cytoplasmic membrane where also very likely the synthesis of the pentaglycine interpeptide takes place. Recently it was shown that lipid II is a peptidoglycan substrate for sortase-catalyzed surface protein anchoring (Perry et al., 2002).

FORMATION OF PENTAGLYCINE INTERPEPTIDE Synthesis of the pentaglycine chain occurs at the membrane-bound lipid II precursor Glc*N*Ac-(β-1,4)-*N*-acetylmuramic acid(-L-Ala-D-*i*Gln-L-Lys-D-Ala-D-Ala)-pyrophosphoryl-undecaprenol by sequential addition of glycine to the ε-amino group of lysine, using glycyl-tRNA as donor, in a ribosome independent fashion (Kamiryo and Matsuhashi, 1972). First hints for an involvement of glycyl-tRNA were based on the finding that incorporation of glycine into polymeric peptidoglycan was prevented by ribonuclease. Under identical conditions, the incorporation of *N*-acetylmuramyl-peptide from the nucleotide precursor was unaffected. Five glycine residues activated by tRNA are added initially to the ε-NH_2 group of lysine and then subsequently to the N-terminus of the growing peptide chain (Kamiryo and Matsuhashi, 1972). This mechanism is in direct contrast to that of protein synthesis, which occurs by addition at the carboxyl terminus. The residues appeared to be added singly with no evidence for the involvement of peptidyl tRNA intermediates. The tRNA of *S. aureus* has been found to contain at least three species of glycyl-tRNA (Gly-tRNA). They can be used for the incorporation of glycine into peptidoglycan, whereas one of the species is inactive in protein synthesis. The formation of all Gly-tRNA is catalyzed by a single Gly-tRNA synthetase, which has been purified (Niyomporn et al., 1968). A more complex situation has been found

in *S. epidermidis* where four different pentapeptide cross-bridges have been shown to be present (Tipper and Berman, 1969). Each contains glycine and L-serine residues in the ratio 3:2 in a characteristic sequence where glycine is always the initial substituent of the ε-amino group of L-lysine. Addition of the amino acids occur at the level of lipid intermediates, the transfer of glycine and serine from their respective tRNAs being catalyzed by membrane-bound enzymes. The incorporation of glycine is independent of that of serine, whereas maximum serine incorporation requires the simultaneous incorporation of glycine. Fractionation of tRNA of *S. epidermidis* has demonstrated the presence of four glycyl- and four seryl-tRNAs. In each case, all species, including one species of each type that did not participate in protein synthesis, were active in cross-bridge synthesis. The glycyl-tRNA with apparent specificity for peptidoglycan synthesis has been purified and shown to be made up of two distinct *iso*-accepting species (Roberts et al., 1974). The two sequences define six spaces and the insertion of an additional base in the dehydrouridine loop of one. Thus, *S. epidermidis* appears to have developed a means of supplying glycine residues for cross-bridge synthesis without involving the machinery of protein synthesis.

THE FEM FACTORS The expression of methicillin resistance depends on several genes named "*fem*" (*f*actors *e*ssential for *m*ethicillin resistance; Berger-Bächi, 1994). Recently, it was demonstrated that FmhB (FemX) links the first glycine residue to the ε-amino group of lysine (Rohrer et al., 1999). Evidence for this function was that, under conditions where *fmhB* is down-regulated, unsubstituted peptidoglycan monomer precursors accumulate. Since FmhB is essential in *S. aureus*, it represents an ideal drug target. While FmhB incorporates the first glycyl residue, FemA incorporates glycyl residues 2 and 3 (Stranden et al., 1997), and FemB, glycyl residues 4 and 5 (Henze et al., 1993). Although FemA and FemB have a relatively high amino acid sequence identity (40%) and similarity (64%), they are specific and cannot substitute for each other. Null mutants of *femAB* are barely viable and depend on compensatory mutations for survival (Ling and Berger-Bächi, 1998). Interestingly, *S. aureus* contains one glycyl-tRNA gene for protein biosynthesis and three non-proteinogenic glycyl-tRNA genes that are involved in cell wall biosynthesis (Green and Vold, 1993). It is tempting to speculate that the two Fem factors and FmhB may preferentially recognize one of the three glycyl-tRNA species. The site of glycine incorporation in the peptidoglycan is shown in Fig. 10.
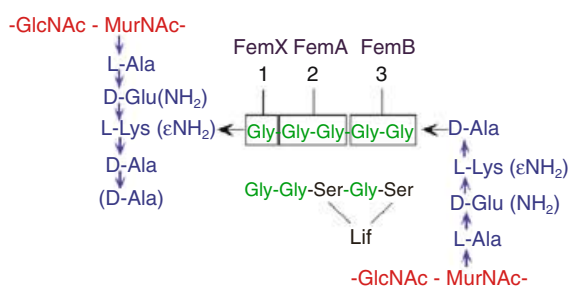
Fig. 10. Pentaglycine biosynthesis of *Staphylococcus aureus* peptidoglycan. Methicillin resistance factor FemX adds the first glycine, FemA adds glycines 2 and 3, and FemB adds glycines 4 and 5. Lif was identified in *S. simulans* var. *staphylolyticus* and is responsible for incorporation of serine residues in positions 3 and 5. Lif, lysostaphin immunity factor; Mur*N*Ac, *N*-acetylmuramic acid; and Glc*N*Ac, *N*-acetylglucosamine.

Other FemAB-like factors have been identified in staphylococci, such as *l*ysostaphin *im*munity *f*actor (Lif) in *S. simulans* biovar *staphylolyticus* (Thumm and Götz, 1997) and Epr in *S. capitis* (Sugai et al., 1998), which protect these strains from their own glycyl-glycine endopeptidase (Thumm and Götz, 1997).

The expression of *lif* in *S. carnosus* led to an increase of the serine-glycine ratio of the interpeptide bridges from 2 to 35%, suggesting that lysostaphin immunity depends on serine incorporation into the interpeptide bridge. If in addition to *lif*, *lss* is coexpressed, the serine-glycine ratio is further increased to 58%, suggesting that Lss selects for optimal serine incorporation. Lif shows similarity to FemA and FemB proteins, which are involved in the biosynthesis of the glycine interpeptide bridge of staphylococcal peptidoglycan. In contrast to Lif, the production of FemA and FemB in *S. carnosus* does not cause lysostaphin immunity (Thumm and Götz, 1997). The putative tRNA[Ser] gene located downstream of *lss* had no recognizable influence on lysostaphin immunity. The *lss* and *lif* genes are flanked by insertion sequences, suggesting that *S. simulans* biovar *staphylolyticus* received *lif* and *lss* by horizontal gene transfer. Recently, it was shown that Lif and Epr lead to incorporation of serine residues into the staphylococcal peptidoglycan interpeptide bridges specifically at positions 3 and 5 and that this incorporation requires the presence of FemA and/or FemB (Ehlert et al., 2000).

TRANSLOCATION OF LIPID II-PENTAGLYCINE In the second stage of the biosynthetic process, the lipid II-pentaglycine is translocated from the inner side of the cytoplasmic membrane to the outer side where the incorporation into the growing peptidoglycan occurs. The initial reaction

involves the transfer of phospho-*N*-acetyl-muramyl-pentapeptide from the nucleotide to undecaprenyl phosphate with the formation of undecaprenyl-P-P-*N*-acetylmuramyl-pentapeptide and UMP. This reaction is catalyzed by a translocase. Incorporation of undecaprenyl-pyrophosphate in UDP-*N*-acetylmuramyl-pentapeptide is inhibited by tunicamycin. In a second step *N*-acetyl-glucosamine is glycosidically linked to the *N*-acetyl-muramyl residue to form undecaprenyl-P-P-*N*-acetylmuramyl (pentapeptide)-*N*-acetylglucosamine and UDP (Anderson et al., 1967). Since these reactions involve both hydrophilic and hydrophobic substrates, it seems likely that both enzymes act at the cytoplasmic lumen of the membrane.

The translocase (phospho-*N*-acetylmuramyl-pentapeptide translocase), which has been extensively studied by Neuhaus and his colleagues (Pless and Neuhaus, 1973), can be conveniently assayed either in the forward direction as a transferase or in the reverse direction, by the exchange reaction following the incorporation of radioactivity from UMP into UDP-*N*-acetyl-muramyl-pentapeptide. The enzyme has been obtained in soluble form from *S. aureus* by treatment of membrane preparations with the nonionic detergent Triton X-100 (Pless and Neuhaus, 1973; Weppner and Neuhaus, 1977). The enzyme is stimulated by the addition of polar lipid fraction containing phosphatidylglycerol. However, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol were equally capable of restoring enzyme activity. Thus, the reactivation of the translocase appeared to result from the provision of a lipid microenvironment rather than a requirement for a specific phospholipid. In vivo, the microenvironment of the translocase is the lipids of the membrane with which it interacts.

Investigation of the microenvironment in membranes of the fluorescent lipid intermediate undecaprenyl-*N*-acetylmuramyl-(*N*-dansyl)-pentapeptide suggests that the lipid intermediate is immobilized within hydrophobic environment close to the membrane surface. This observation is supported by the earlier finding that spin-labelled lipid intermediate could still form complexes with vancomycin and ristocetin (Johnston and Neuhaus, 1975). It was proposed that at least the terminal D-alanyl-D-alanine-dipeptide of the pentapeptide side chain has access to the aqueous phase. Vancomycin and ristocetin mimics the effect of detergents. At low concentrations, the antibiotic stimulates the transfer but not the exchange reaction. Hammes and coworkers (Hammes and Neuhaus, 1974) have investigated the specificity of phospho-*N*-acetylmuramyl-pentapeptide translocase from *S. aureus*. They could show that the translocase

has 24% activity when the terminal D-alanine is missing, 60% activity when the terminal amino acid is glycine instead of D-alanine, and 57% activity when *meso*-diaminopimelic acid (mA$_2$pm) instead of L-lysine is present.

In many organisms, the lipid-linked disaccharide pentapeptide units are utilized directly for synthesis of peptidoglycan. In *S. aureus*, the carboxyl group of D-isoglutamic acid is amidated. The reaction is catalyzed by a membrane-bound enzyme. The enzyme can utilize either glutamine or NH$_4^+$, and both undecaprenyl-P-P-*N*-acetyl-muramyl-pentapeptide and disaccharide-pentapeptide act as substrates, whereas a nucleotide precursor UDP-*N*-acetylmuramyl-pentapeptide is completely inactive. The reaction involves hydrolysis of ATP to ADP. It seems likely that many other minor differences found in the structure of peptidoglycan such as the O-acetylation of muramic acid residues in *S. aureus* (Ghuysen and Hakenbeck, 1994) and the addition to the peptide side chain of single amino acid residues, not involved in interpeptide-bridge formation, occurs at the level of lipid intermediates.

The final reaction of the membrane-mediated stage of peptidoglycan synthesis is also the initial reaction in the third stage, i.e., the incorporation of the newly synthesized disaccharide peptide unit into the growing peptidoglycan. The presence of uncross-linked side chains in all peptidoglycans examined argues strongly that polymerization occurs by extension of the glycan chain (transglycosylation) prior to peptide-bond formation (transpeptidation). The finding of free reducing groups of muramic acid in the walls of *S. aureus* (Ward and Perkins, 1973) suggests that there must exist some mechanisms to terminate synthesis of a particular glycan chain by detaching the lipid carrier. At present, the nature of this process is not evident.

TRANSPEPTIDATION: THE FORMATION OF CROSSLINKS By the mid-1960s, detailed studies of the chemical structure of several peptidoglycans established the presence of crosslinked peptides. It was suggested that D-alanyl-D-alanine-dipeptide appeared to be involved in the formation of these cross-linkages. When sublethal concentrations of penicillin were applied, the walls contained an increase in the free amino groups of glycine and an excess of alanine over those walls prepared from organisms grown in the absence of penicillin. These observations were independently confirmed by direct chemical analysis of staphylococcal walls prepared from organisms grown under similar conditions (Tipper and Strominger, 1968). In the presence of penicillin, an increased synthesis of uncross-

linked peptide monomer units was observed. These units retained the complete D-alanyl-D-alanine terminus of the nucleotide precursor and were substituted with the pentaglycine cross-bridge peptide on the ε-amino group of L-lysine. During subsequent growth of the organism in the absence of penicillin, these monomer units did not become crosslinked. To explain these observations, it was proposed that incorporation of the newly synthesized disaccharide-peptide occurred at the wall-membrane interface with almost immediate formation of crosslinks to adjacent peptide side chains. In staphylococci, units synthesized in the presence of penicillin could not form crosslinks on the subsequent removal of the antibiotic because continued synthesis of linear uncross-linked peptidoglycan during the initial penicillin treatment moved these units outside the reach of the transpeptidase molecules. These in vivo studies supported the conclusion that crosslinkage in peptidoglycan occurred by means of a transpeptidation reaction inhibited by β-lactam antibiotics.

In vitro, cross-link studies with cell wall preparations from *S. aureus* (Mirelman and Sharon, 1972) were shown to retain some cytoplasmic membrane not removed by extensive washing. These preparations catalyzed the incorporation of radioactivity from UDP-*N*-acetylmuramyl([^{14}C]-L-lysine)pentapeptide into both peptidoglycan and lipid intermediates. The penicillin sensitive release of D-alanine occurred when the wall-membrane preparation was incubated with the two nucleotide precursors and glycine, whereas no such release occurred in the preparations incubated in the absence of UDP-*N*-acetylglucosamine. Glycine incorporation was sensitive to ribonuclease. Penicillin also inhibited the incorporation of glycine, but in contrast to release of D-alanine, this was never complete.

AUTOLYSINS AND TURNOVER OF BACTERIAL WALL POLYMERS There are various autolytic enzymes present in most staphylococci:

1) An enzyme that hydrolyzes the *N*-acetyl-muramyl-1,4-β-*N*-acetylglucosamine bonds in the glycans to liberate free-reducing groups of *N*-acetylmuramic acid; since this enzyme is only active with micrococcal but not with staphylo-coccal peptidoglycan, we postulate that it plays a role before the peptidoglycan becomes O-acetylated

2) A β-*N*-acetylglucosaminidase that liberates the free reducing groups of *N*-acetylglucosamine

3) An *N*-acetylmuramyl-L-alanine amidase (amidase) that hydrolyzes the bond between the glycan chains and the peptide subunits

4) Endopeptidases that can hydrolyze some of the main peptides and the bridge peptide when they occur between the C-terminal D-alanine and the amino group of a contiguous peptide chain

5) Commonly occurring in many organisms are D-alanine-carboxypeptidases that can cleave off any carboxyl terminal D-alanyl residues of the peptidoglycan. These enzymes, however, are not known to act as autolysins and therefore will not be considered.

Staphylococcus aureus does not exhibit a lysozyme like muramidase (EC 3.2.1.17), which cleaves its O-acetylated peptidoglycan.

Pulse chase studies indicated that walls of many organisms such as bacilli, lactobacilli or staphylococci are in a state of flux. Wall synthesis and degradation are balanced by a so-called "turnover of the cell wall." If wall synthesis is inhibited in such situations, without a corresponding inhibition of either the formation or action of the autolysins, cell lysis might be expected and indeed occurs. Antibiotics that inhibit wall synthesis are bactericidal, unlike most of those inhibiting protein synthesis, which are bacteriostatic.

There are still several open questions: Is the inhibition of wall synthesis sufficient to kill bacteria? Do cells lyse because their walls can no longer expand so that the cell bursts? Is autolytic action essential? One possible way to answer these questions would be to stop the formation of autolysins by blocking protein synthesis. A combination of chloramphenicol and penicillins is not bactericidal for *E. coli* or for staphylococci (Rogers et al., 1980b). Lysis of staphylococci could also be stopped, as with *B. subtilis* and *Enterococcus faecalis*, by the inhibition of protein synthesis. However, to date the answers to these questions have been inconclusive. The observation that an autolytic deficient strain of *S. aureus* failed to show wall turnover, although the wildtype turnover rate was rather slow (Chatterjee et al., 1976) should be reevaluated.

Suginaka et al. (1979) have shown that the addition of homologous lipoteichoic acid to cultures of *S. aureus* treated with penicillin prevents lysis and death of the bacteria. Forssman antigen (lipoteichoic acid from pneumococci) can also prevent penicillin-induced lysis in *S. aureus*. Lipoteichoic acids or lipids certainly regulate the action of autolysins in vivo. It was also found that wall autolysis is inhibited by sodium polyanethole sulfonate "liquoid" (Wecke et al., 1986). The inhibition is not due to direct interaction with the autolysins, and liquoid blocks very likely the target structures of autolysins, especially the wall teichoic acid (WTA).

BIOLOGICAL ACTIVITY OF PEPTIDOGLYCAN It was early recognized that cell wall muramyl peptides exert excellent adjuvant properties, and consequently a variety of muramyl derivatives with biological potentials as adjuvant have been synthesized and investigated (Adam et al., 1975). In a somewhat analogous manner, another interesting biological property (i.e., the somnogenic effects of muramyl compounds) was investigated by Krueger and coworkers (Johannsen et al., 1989; Johannsen et al., 1991a; Johannsen et al., 1991b). It is of interest that macrophages produce somnogenic and pyrogenic muramyl peptides during digestion of staphylococci. Somnogenic compounds of bacterial origin are obviously small enough to cross the blood-brain barrier, and it is of interest to speculate that lysozyme capable of digesting the insoluble wall peptidoglycan polymer could have a role in the production of suitably sized substances with such properties.

Obviously the immune system recognizes that muramyl peptides are products of bacteria, and responds by becoming activated to resist infection. This resistance to infection is nonspecific and extends to unrelated species of bacteria, fungi, and viruses. A key mechanism of the resistance to infection is activation of macrophages. Macrophage activation results in increased production of microbicidal oxygen radicals like superoxide and peroxide, and in increased secretion of inflammatory cytokines like interleukin-1β and tumor necrosis factor-α. These cytokines, besides activating neutrophils, B lymphocytes, and T lymphocytes, act on the central nervous system to induce physiological responses like fever and sleep. These physiological responses also aid in combating infection. Muramyl peptides activate macrophages and other cells of the immune system to kill cancer cells. It is therefore believed that muramyl peptides and similar agents will become more important as therapeutic agents in the future, owing to increasing resistance of microbes to antibiotics, and increasing numbers of patients with immunodeficiencies (Pabst et al., 1999).

Mammals have a peptidoglycan recognition protein (PGRP) which binds to peptidoglycan (PG) or live bacteria and which is upregulated by PG. This PGRP is ubiquitous and involved in innate immunity. Tag7, a novel cytokine, is also induced by bacterial products; Tag7 is apoptotic to murine L929 cells in a NF-κB-independent manner. Both the *PGRP* and *tag7* genes are expressed in brain, lymphatic and hematopoietic tissues. Recently, it was found that murine *PGRP* and *tag7* encode identical transcripts, and their corresponding proteins have structural relationships to lysozymes. Furthermore, the cDNA of rat PGRP was cloned and expressed in brains of sleep-deprived and control rats. It turned out that PGRP expression was upregulated by sleep deprivation, suggesting a role for PGRP in a homeostatic regulation of sleep (Rehman et al., 2001).

Antibiotics Affecting Bacterial Peptidoglycan Synthesis

PHOSPHONOMYCIN (FOSFOMYCIN) This antibiotic, the isolation and characterization of which were first described in 1969, inhibits what might be regarded as the earliest step in peptidoglycan synthesis. It inhibits the transfer of the enolpyruvate residue from phosphoenol pyruvate to UDP-*N*-acetylglucosamine (UDP-*N*-acetylglucosamine-3-*O*-enolpyruvyl transferase). Phosphonomycin binds covalently to cysteine residues of the transferase. Resistance is achieved by mutations in the transport systems. For review of the cell wall antibiotics, see Rogers et al. (1980a).

CYCLOSERINE, O-CARBAMOYL-D-SERINE, ALAPHOSPHIN, AND THE HALOALANINES Antibiotics that inhibit D-alanine metabolism are cycloserine, O-carbamoyl-D-serine, alaphosphin (L-alanyl-L-1-aminoethyl phosphonic acid) and the haloalanines. Alanine racemase is competitively and irreversibly inhibited by cycloserine, O-carbamoyl-D-serine and the haloalanines.

The inhibiting effect of D-cycloserine on bacterial growth can be reversed by the addition of D-alanine. The presence of D-alanine prevents conversion to spheroplasts of several bacteria incubated with cycloserine in osmotically stabilized media. Cycloserine acts by inhibiting the alanine racemase and thus the incorporation of D-alanine into the peptidoglycan. As a consequence, UDP-*N*-acetylmuramyl-L-alanine-D-isoglutamyl-L-lysine accumulates. O-carbamoyl-D-serine, alaphosphin (L-alanyl-L-1-aminoethyl phosphonic acid) and the haloalanines act in a similar way. While D-cycloserine alone appears to inhibit D-alanine:D-alanine ligase, the D-alanyl-D-alanine adding enzyme (UDP-*N*-acetylmuramyl-L-alanine-D-isoglu-L-lys:D-Ala-D-Ala ligase [ADP]) is not affected by any of these antibiotics. D-Cycloserine is a competitor of the substrate for both alanine racemase and D-alanine:D-alanine ligase. The inhibition constant of D-cycloserine for alanine racemase is 5×10^{-5}. Resistance to D-cycloserine can arise either by elevated racemase and ligase activity or by inability to transport the antibiotic.

BACITRACIN Bacitracin specifically inhibits the dephosphorylation of undecaprenyl-pyrophosphate.

T<small>UNICAMYCIN</small> Initially it was observed that tunicamycin inhibits glycoprotein synthesis in Newcastle disease virus-infected cells (Takatsuki and Tamura, 1971). In bacteria, it inhibits the phospho-*N*-acetylmuramyl-pentapeptide translocase, i.e., the formation of the first lipid intermediate in peptidoglycan synthesis. In *S. aureus* and several bacilli, it inhibits also the linking of WTA to peptidoglycan (Bracha and Glaser, 1976; Bracha et al., 1978).

V<small>ANCOMYCIN AND</small> R<small>ISTOCETIN</small> B Inhibition occurs very rapidly and binding of the antibiotic to bacteria takes place within 20 seconds of treatment. In *S. aureus*, approximately 10^7 molecules are bound per cell (although this number might be too high because of aggregate formation). Both vancomycin and ristocetin B bind preferentially to the terminal D-alanyl-D-alanine peptide of the UDP-*N*-acetylmuramyl-pentapeptide. The binding site of ramoplanin appears to be different from that of vancomycin (Nieto et al., 1972; Somner and Reynolds, 1990).

Glycopeptide resistance in *S. aureus* is poorly understood. Characterization of vancomycin resistant *S. aureus* (VRSA) strains from all over the world confirmed that emergence of vancomycin resistance in *S. aureus* is a global issue. A certain group of *S. aureus*, designated "hetero-VRSA," frequently generates VRSA upon exposure to vancomycin, and is associated with infections that are potentially refractory to vancomycin therapy (Hiramatsu, 2001). Vancomycin resistance is acquired via mutation and cell wall thickening, due to accumulation of excess amounts of peptidoglycan. This seems to be a common resistance mechanism for all VRSA strains isolated so far (Geisel et al., 2001; Komatsuzawa et al., 2002). By exposure to vancomycin, a resistant *S. aureus* COL mutant was isolated. This mutant showed decreased susceptibility to teicoplanin (8-fold), methicillin (2-fold), macarbomycin (8-fold), and moenomycin (16-fold). Macarbomycin and moenomycin are thought to directly inhibit transglycosylase activity. Characterization of the mutant revealed a thickened cell wall and suppression of penicillin-induced lysis, although the amounts of the five penicillin-binding proteins (PBPs 1, 2, 3 and 4) and MecA, and the profiles of peptidoglycan hydrolases were not altered. Analysis of muropeptide profile and glycan chain length distribution by reverse-phase high-pressure liquid chromatography revealed slightly decreased peptide crosslinking and an increased average glycan chain length compared to those of the parent. These results together suggest that a transglycosylase activity was enhanced in the mutant. This may represent a novel mechanism of glycopep-

tide resistance in *S. aureus* (Komatsuzawa et al., 2002).

In June 2002, a VRSA was isolated from a swab obtained from a catheter exit site of a Michigan resident aged 40 years with diabetes, peripheral vascular disease, and chronic renal failure. The patient received dialysis at an outpatient facility (Centers for Disease Control and Prevention, 2002). In June, the patient developed a suspected catheter exit-site infection, and the temporary dialysis catheter was removed; cultures of the exit site and catheter tip subsequently grew *S. aureus* resistant to oxacillin (minimal inhibitory concentration [MIC] > 16 µg/ml) and vancomycin (MIC > 128 µg/ml). It turned out that the isolate contained the *vanA* vancomycin resistance genes from enterococci, which is consistent with the glycopeptide MIC profiles. Transfer of *vanA* genes from *Enterococcus faecalis*, with which the patient was also infected, to *S. aureus* is strongly indicated. And to make it worse, the *S. aureus* isolate contained in addition the oxacillin/methicillin-resistance gene *mecA*. This report describes the first clinical isolate of *S. aureus* that is fully resistant to vancomycin (Centers for Disease Control and Prevention, 2002). Transfer of *van* resistance genes from enterococci to *S. aureus* has long been predicted, with such a conjugative transfer having been demonstrated in vitro (Noble et al., 1992).

β-L<small>ACTAM AND</small> M<small>ETHICILLIN</small> R<small>ESISTANCE</small> Three basic enzymes have been described which are subject to penicillin-inhibition: transpeptidases, D-alanine carboxypeptidases, and endopeptidases. The blanket resistance of methicillin-resistant *S. aureus* to all β-lactam antibiotics is related to the properties of the key component of this resistance mechanism: the "acquired" penicillin-binding protein MecA, which has unusual low affinity for all β-lactam antibiotics. Until now, the accepted model of resistance had implied that in the presence of β-lactam antibiotics, MecA must take over the biosynthesis of staphylococcal cell wall from the four native staphylococcal PBPs (PBP1, PBP2, PBP3 and PBP4) because the latter become rapidly acylated and inactivated at even low concentrations of the antibiotic. However, recent observations indicate that this model requires revision. Inactivation of the transglycosylase domain, but not the transpeptidase domain, of MecA of *S. aureus* prevents expression of β-lactam resistance, despite the presence of the low-affinity MecA. The observations suggest that cell-wall synthesis in the presence of β-lactam antibiotics requires the cooperative functioning of the transglycosylase domain of the native staphylococcal PBP2

and the transpeptidase domain of the MecA (Pinho et al., 2001). There are indications that the *mecA* gene was acquired from *S. sciuri* which is, however, uniformly susceptible to β-lactam antibiotics. Recently, it was shown that the *mecA* homologue in *S. sciuri* is hardly expressed but is well expressed by a single mutation in the promoter region and provides methicillin resistance in a susceptible *S. aureus* strain. This supports the proposition that the *mecA* homologue ubiquitous in the antibiotic-susceptible animal species (*S. sciuri*) may indeed be an evolutionary precursor of the methicillin resistance gene *mecA* of the pathogenic strains of MRSA (Wu et al., 2001).

The peptidoglycan isolated from methicillin resistant *S. aureus* (MRSA) strains that contain *mecA* did not differ from that of the susceptible *S. aureus* isolates, suggesting that the activity of MecA cannot be distinguished from that of existing penicillin binding proteins (PBPs), or that it is not functioning in cell wall biosynthesis, as long as these strains are cultivated in the absence of β-lactam antibiotics (de Jonge et al., 1992; Labischinski and Maidhof, 1994). When β-lactam antibiotics saturate the normal set of staphylococcal PBPs, the cell wall is still produced due to the presence of MecA but is drastically hypocrosslinked. The expression of methicillin resistance does depend on *fem* genes (Berger-Bächi, 1994). The inactivation of *femA* and *femB* results in loss of resistance of MRSA strains and induction of hypersensitivity of sensitive staphylococci.

ACQUISITION OF *mecA* AND ITS REGULATION Both annotated *S. aureus* N315 and Mu50 genomes contain a so-called "staphylococcal cassette chromosome mec" (SCCmec) which encodes resistance to β-lactams, bleomycin, macrolide-lincosamide streptogramin B, aminoglycosides (tobramycin and amikacin), and spectinomycin (Kuroda et al., 2001). One can also consider the 40–60-kb long SCCmec as a resistance island, which was acquired very likely by horizontal gene transfer.

In opposite orientation to *mecA* are two co-transcribed genes, *mecR1* and *mecI*. The *mecR1* gene encodes a membrane-bound signal transduction protein (MecR1), while *mecI* encodes a transcriptional regulator (MecI). Between *mecA* and *mecR1* are the promoters for these genes and an operator region that encompasses the –10 sequence of *mecA* and the –35 sequence of *mecR1* (Sharma et al., 1998). MecR1 and MecI have high protein sequence homology with the proteins, BlaR1 and BlaI, respectively, that are involved in the inducible expression of the plasmid-mediated staphylococcal β-lactamase gene, *blaZ*. MecI is a tight regulator of *mecA*

transcription, and most β-lactam antibiotics do not efficiently activate MecR1 (Kuwahara-Arai et al., 1996). Consequently, some isolates, referred to as "pre-MRSA," are methicillin-sensitive despite carrying the *mecA* gene. However, selective pressure through antibiotic usage has promoted *S. aureus* isolates that have mutations or deletions in *mecI* or the *mecA* promoter/operator region, giving rise to an inactive repressor and constitutive *mecA* expression. These mutants can display homogeneous (all cells are resistant to high concentrations of methicillin [>128 µg/liter]) or heterogeneous (only a small minority of cells exhibit high-level methicillin resistance) methicillin resistance phenotypes (Kondo et al., 2001).

Other Cell Wall Components

CELL WALL TEICHOIC ACID The staphylococcal cell wall consists not only of a thick peptidoglycan fabric but also of polymers of alternating phosphate and alditol groups called "teichoic acids." The discovery of the teichoic acids resulted from the identification of ribitol in a cytidine nucleotide isolated and characterized by Baddiley and coworkers already in the mid-1950s. The size of ribitol teichoic acids and the nature of their linkage to glycosaminopeptides was described in 1965 (Hay et al., 1965).

There are two teichoic acid types in staphylococci: a wall teichoic acid (WTA), which is covalently bound to the peptidoglycan and which is, depending on the species, composed of ribitol and/or glycerol teichoic acids (Pooley and Karamata, 1994). The WTA is distinct from the subsequently discovered lipoteichoic acids (LTAs). LTA contains only glycerol phosphate and is anchored in the cytoplasmic membranes of Gram-positive bacteria (Fischer, 1988). The alditol units of WTAs and the LTAs contain normal substituents of which D-alanine and glycosyl residues are most common. The structures of the *S. aureus* WTA and LTA are shown in Fig. 11.

STRUCTURE AND BIOSYNTHESIS OF WALL TEICHOIC ACID The cell wall teichoic acid structures of 13 staphylococcal type strains were determined (Endl et al., 1983). Most of the strains contain a poly(polyolphosphate) teichoic acid with glycerol and/or ribitol as polyol component. The polyolphosphate backbone is partially substituted with various combinations of sugars and/or amino sugars. Most of the substituents occur in a monomeric form, but some strains also contain dimers of *N*-acetylglucosamine as substituents. *Staphylococcus hyicus* subsp. *hyicus* and *S. sciuri* revealed rather complex cell wall teichoic acids. They consist of repeating sequences of phos-
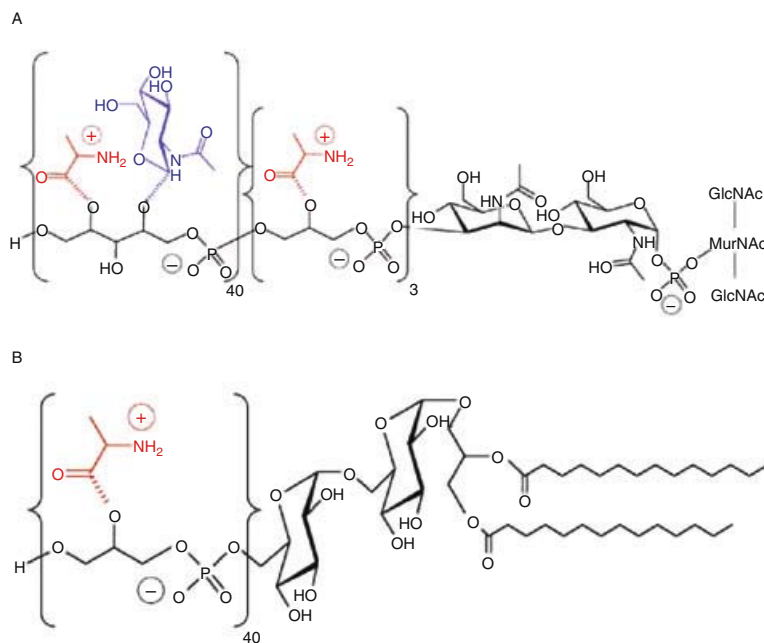
Fig. 11. A) Structure of the *S. aureus* wall teichoic acid (WTA). The WTA is linked via a phosphodiester bond to the 6 OH-group of muramic acid residue in the glycan strand. 1,3-Man*N*Ac-Glc*N*Ac-PP-prenol serves as substrate. The 2 OH-group of Man*N*Ac is phosphodiester linked with three units of D-alanylated glycerol-phosphates to which approximately 40 units of ribitol-phosphates (substituted with D-alanine and/or Glc*N*Ac) are linked. B) Structure of the *S. aureus* lipoteichoic acid (LTA). The membrane anchor region is a diacylglycerol which is C1 linked to the disaccharide Glc(ß1,6); in the second glucosyl residue of the disaccharide, approximately 40 units of glycerol phosphates are linked via the 6 OH-group. Position 2 of the glycerol phosphate residues is in part substituted either with D-alanine or D-alanine ester and glycosyl (mostly Glc*N*Ac) residues. Man*N*Ac, *N*-acetylmannosamine; Glc*N*Ac, glucosamine; PP, pentose phosphate. Structure was drawn by Peschel (2002).

phate-glycerol-phosphate-*N*-acetylglucosamine. The amino sugar component is present in this case as a monomer or an oligomer. Moreover, the glycerol residues are partially substituted with *N*-acetylglucosamine. The cell wall teichoic acid of *S. auricularis* is a poly(*N*-acetylglucosaminyl-phosphate) polymer similar to that found in *Macrococcus caseolyticus*.

Biosynthesis of ribitol teichoic acids was studied using membrane fractions of *S. aureus* H (Yokoyama et al., 1986). Incubation of *S. aureus* membranes with CDP-glycerol and Man*N*Ac-[14C]Glc*N*Ac-PP-prenol led to synthesis of (glycerol phosphate)-1-3-Man*N*Ac-[14C]Glc*N*Ac-PP-prenol. In *S. aureus* (glycerol phosphate)-2-Man*N*Ac-[14C]Glc*N*Ac-PP-prenol as well as (glycerol phosphate)-3-Man*N*Ac-[14C]Glc*N*Ac-PP-prenol served as an acceptor for ribitol phosphate units, but (glycerol phosphate)-Man*N*Ac-[14C]Glc*N*Ac-PP-prenol did not. The covalent attachment of WTA to peptidoglycan does not appear to play a role in the maintenance of the shape or rigidity of the cell wall, since their extraction with trichloracetic acid (TCA) leaves an essentially intact residual wall structure (Baddiley, 1972). While the cell-biological functions of

LTA are manifold, not much is known about WTA, which however plays an important role as bacteriophage receptor (Schleifer and Steber, 1974). It was for a long time an open question whether WTA is essential for growth. Recently in the group of A. Peschel, a completely WTA negative deletion mutant was isolated which exhibits normal growth but became resistant to a number of bacteriophages was impaired in adherence to nasal cells, and was completely unable to colonize cotton rat nares (Weidenmaier et al., 2004).

LIPOTEICHOIC ACID (LTA) With a pure (>99%) LTA preparation from *S. aureus*, nuclear magnetic resonance and mass spectrometry analyses were carried out (Morath et al., 2001). The results essentially corroborate the structure described by Fischer (1994). The average chain length of polyglycerol phosphate was 45–50 units; 70% of the glycerophosphate units were esterified with D-alanine, 15% bore α-D-*N*-acetylglucosamine, and 15% had no substituents. The fatty acids had an average chain length of $C_{15}H_{31}$. Fischer found that the alanine esters are rapidly lost from completed LTA and he hypoth-

esized that by spontaneous hydrolysis of the labile ester bond, D-alanine is transferred to the WTA. The D-alanine loss in LTA is compensated by incorporation of new alanine ester at a rate adjusted to the velocity of loss (Fischer, 1994).

Biological Functions It has been proposed (Heptinstall et al., 1970) that teichoic acids (which are highly negatively charged polymers) play an important role in divalent cation sequestration at the bacterial surface. Indeed it was found that the alanine ester content influences the magnesium binding capacity of walls of *S. aureus* H grown at different pH values (Archibald et al., 1973).

Fischer and coworkers investigated the influence of the degree of D-alanine esterification of staphylococcal LTA on autolysin activity using extracellular autolysin from *S. aureus* (Fischer et al., 1981). It turned out that the inhibitory activity was highest with D-alanine-free LTA. Glycosylation of LTA up to an extent of 0.5 did not depress inhibitory activity. They hypothesized that the anti-autolytic activity of LTA resides in a sequence of glycerophosphate units and that the negative charges of appropriately spaced phosphodiester groups play a crucial role. This work shows that autolysis is regulated in vivo by the alanine ester content of the LTA, and very likely by the WTA too, which also carries ester bound D-alanine. LTA is also an important antigen (Knox and Wicken, 1973). Some of the biological properties of the staphylococcal envelope components are listed in Table 13.

Table 13. Some biological properties of staphylococcal envelope components.

Envelope components	Property or activity
Peptidoglycan	Cell shape
	Osmotic stability
	Antigenic
	Pyrogenic
Peptidoglycolipid	Adjuvant
Muramylpeptides	Somnogenic
WTA	Antigenic
	Phage receptor
	Divalent cation binding
	Autolysis inhibition/regulation
LTA	Immunogenic
	Cell adhesion
	Phage receptor
	Complement activation
	Autolysis inhibition/regulation
Lipoprotein	Cell binding
	Immunogenic
	Mitogenic

Abbreviations: WTA, wall teichoic acid; and LTA, lipoteichoic acid.
[a]From Salton (1994).

Apart from lethal toxicity and pyrogenicity, LTA appeared to share many of the biological properties exhibited by lipopolysaccharides (Wicken and Knox, 1980). Critical micelle concentrations of various LTAs has been determined to be 1–10 μg/liter, suggesting that acylated LTAs in their monomer forms may represent the major configuration of extracellular LTAs in bacterial culture fluids (Wicken et al., 1986).

Great care has to be taken when analyzing commercial LTA preparations for immune stimulating activity. One must be aware that frequently these preparations are inhomogeneous and contain decomposed LTA and endotoxins of more than 10 ng of LPS/mg of LTA (Morath et al., 2002). A novel isolation procedure has been worked out that leads to a pure (> 99%) biologically active LTA, allowing the first structural analysis by nuclear magnetic resonance and mass spectrometry. A comparison with LTA purified by standard techniques revealed that alanine substituents are lost during standard purification, resulting in attenuated cytokine induction activity. In line with this finding, hydrolysis of alanine substituents of active LTA eliminated cytokine induction. Purified *S. aureus* LTA induced the release of TNF-α, IL-1β, IL-6 and IL-10 in human whole blood. Soluble CD14 (sCD14) inhibited monokine induction by LTA but failed to confer LTA responsiveness for IL-6 and IL-8 release of human umbilical vein endothelial cells (HUVECs). In a competitive LPS-binding protein (LBP) binding assay, the IC_{50} of the tested LTA preparations was up to 3230-fold higher than for LPS. The LBP enhanced TNF-α release of human peripheral blood mononuclear cells (PBMCs) upon LPS but not LTA stimulation. These data demonstrate a differential role for the serum proteins LBP and sCD14 in the recognition of LPS and LTA (Hermann et al., 2002). There is evidence that the recognition sites of CD14 for LPS and LTA are distinct with a partial overlap. While the maximal achievable monokine release in response to LTA or to LPS was comparable, all LTA induced significantly less IL-12 and IFN-γ. IL-12 substitution increased LTA-inducible IFN-γ release up to 180-fold, suggesting a critical role of poor LTA-inducible IL-12 for IFN-γ formation. Pretreatment with IFN-γ rendered galactosamine-sensitized mice sensitive to challenge with LTA. When compared to LPS, LTA (a major immunostimulatory component of *S. aureus*) is a weak inducer of IL-12 and subsequent IFN-γ formation, which might explain the lower toxicity in vivo.

In a recent study, the effects of LPS from *E. coli*, LTA and peptidoglycan (PG) from *S. aureus* and live *S. aureus* on leukocyte-endothelial interactions in vivo have been investigated (Yipp et

al., 2002). It was found that local administration of LPS into muscle induced significant leukocyte rolling, adhesion, and emigration in postcapillary venules at the site of injection. Given systemically, LPS caused circulating leukocyte counts to drop dramatically and neutrophil counts to increase in the lung. However, the drop in circulating leukocytes was not associated with leukocyte sequestration to the site of injection (peritoneum) or to peripheral microvessels in muscles. Unlike LPS, LTA had no systemic and very minor local effect on leukocyte-endothelium interactions, even at high doses and prolonged exposure. LPS, but not LTA, potently activated human endothelium to recruit leukocytes under flow conditions in vitro. Endothelial adhesion molecule expression was also increased extensively with LPS, but not LTA. Interestingly, systemic administration of live *S. aureus* induced leukocyte-endothelial cell responses similar to LPS. PG was able to induce leukocyte-endothelial interactions in muscle and peritoneum, but had no effect systemically (no increase in neutrophils in lungs and no decrease in circulating neutrophil counts). These results demonstrate that: 1) LPS has potent, but divergent local and systemic effects on leukocyte-endothelial interactions; and 2) *S. aureus* can induce a systemic response similar to LPS, but this response is unlikely to be due to LTA, but more likely to be mediated in part by PG.

Toll-like receptors (TLRs) are involved in cellular activation by microbial products, including lipopolysaccharide, lipoproteins, and peptidoglycan. Although for these ligands the specific transmembrane signal transducers TLR-4, TLR-2, or TLR-2 and -6 have now been identified, the molecular basis of recognition of LTA and related glycolipids has not been completely understood. Purified LTAs from *S. aureus* and *B. subtilis* exhibited TLR-2 dependence in nuclear factor κB activation and cytokine induction. The signaling molecules MyD88 and NIK appear to be also involved in cell stimulation by LTA (Opitz et al., 2001). The results presented here suggest that TLR-2 is the main receptor for the LTA-mediated inflammatory response.

Alanine Esterification and Virulence Staphylococcus aureus and *S. xylosus* normally tolerate high concentrations of several positively charged antimicrobial peptides. Andreas Peschel isolated staphylococcal transposon mutants that were not only hypersensitive to lantibiotics such as gallidermin (Götz and Jung, 2001a) but also to human defensin HNP1- 3, animal-derived protegrins, and other antimicrobial peptides (Peschel et al., 1999). All these substances are *c*ationic *a*nti*m*icrobial *p*eptides (CAMPs), many of which have membrane-damaging activity.

One mutant type lacked ester-bound D-alanine in the teichoic acids of both WTA and LTA. As a result the cells carry an increased negative surface charge and bound fewer anionic, but more positively charged peptides and proteins (Peschel et al., 1999). In addition, they became much more sensitive to human defensins, animal-derived protegrins, tachyplesins, and magainin II, and to the lantibiotics gallidermin and nisin. The mutation was located within the *dlt* genes involved in the transfer of D-alanine into teichoic acids. The fact that high concentrations of magnesium ions compromised the protective properties of D-alanine esters corroborates the hypothesis that the increased negative charge of the cell wall is responsible for the hypersensitivity against positively charged antimicrobial peptides. These results also show that the D-alanine-esterification of teichoic acids (which occurs in many Gram-positive bacteria) is not essential for growth but is essential in the protection against human and animal defense systems (Peschel et al., 1999). The innate immune system is largely based on the release of defensins by immune cells and various tissue cells such as epithelial or endothelial cells. Therefore, *dltA* mutants should be less virulent than wild type cells. Indeed it was demonstrated that the *S. aureus dltA* mutant lacking D-alanine modifications of teichoic acids is highly susceptible to neutrophil killing and is virulence-attenuated in mice (Collins et al., 2002). These results clearly show that D-alanine-esterification of teichoic acids is a virulence factor.

Substances such as defensins from the granules of phagocytes, epithelial surfaces, and skin (Ganz and Lehrer, 1994) share an amphiphilic cationic structure and a membrane-damaging activity by forming pores or disintegrating the cytoplasmic membrane bilayer. They are not only produced by mammals but also by insects and bacteria (Sahl and Bierbaum, 1998). CAMPs represent an important human defense mechanism, protecting skin and epithelia against invading microorganisms and assisting neutrophils and platelets. *Staphylococcus aureus* and other bacterial pathogens have evolved countermeasures to limit the effectiveness of CAMPs, including the repulsion of CAMPs by reducing the net negative charge of the bacterial cell envelope through covalent modification of anionic molecules (e.g., teichoic acids, phospholipids and lipid A). Mutants susceptible to CAMPs are more efficiently inactivated by phagocytes and are virulence-attenuated, indicating that CAMP resistance plays a key role in bacterial infections as reviewed by Peschel (2002).

Another biological effect of the *S. aureus dltA* mutant was that it became biofilm-negative. The underlying mechanism is its attenuated adher-

ence to artificial surfaces that play a key role in the first step of biofilm formation. The lack of D-alanine esters with the associated increase of negative surface charge affected colonization of polystyrene or glass (Gross et al., 2001), while PIA (polysaccharide intercellular adhesion) production, necessary for accumulated growth was only marginally influenced in the mutant. The data suggest that repulsive electrostatic forces can lead to reduced staphylococcal biofilm formation. Interestingly, the addition of >10 mM $MgCl_2$ but not $CaCl_2$ completely restored biofilm formation, again corroborating the important role of teichoic acids in scavenging Mg^{+2} ions.

Cell Wall Bound Proteins

Many surface proteins of Gram-positive bacteria are anchored to the cell wall envelope by a transpeptidation mechanism, requiring a C-terminal sorting signal with a conserved LPXTG motif   (leucine-proline-unknown-threonine-glycine). Pioneering work in the identification and characterization of the enzyme involved in protein anchoring and the underlying mechanism was carried out in the group of Olaf Schneewind. In a recent review, the main achievements are summarized (Mazmanian et al., 2001).

PROTEIN A—A CELL WALL-ANCHORED SURFACE PROTEIN   Protein A, an immunoglobulin G (IgG) binding protein, was the first identified surface protein of *S. aureus* (Sjödahl, 1977). Protein A is covalently bound via its C-terminal end to the pentaglycine interpeptide bridge of peptidoglycans and can be released by treatment with lysostaphin, a glycyl-glycine endopeptidase. Protein A (Spa) was one of the first proteins shown to be covalently anchored to the peptidoglycan. Protein A is synthesized as a precursor, containing an N-terminal signal peptide, a pro-peptide region, five IgG-binding domains (Moks et al., 1986), a cell-wall spanning region, and finally a 35-residue cell wall sorting (cws) sequence which is conserved in surface anchored proteins of most Gram-positive bacteria (Schneewind et al., 1992). The cws consists of the typical LPXTG motif, which is followed by a hydrophobic domain and a tail of mostly positively charged residues. Mutations at the LPXTG motif lead normally to the secretion of the mutant protein into the extracellular medium. Deletion of this motif prevents cleavage and cell wall anchoring of protein A. The cws sequence of protein A is representative (Fig. 12).

Apart from *spa*, there are in *S. aureus* several other proteins with a typical cws sequence (Table 14).

SORTASE   Sortase (SrtA) cleaves polypeptides between the threonine and the glycine of the LPXTG motif (peptidase reaction) and catalyzes the formation of an amide bond between the carboxyl-group of threonine and the amino group of peptidoglycan crossbridges (transpeptidation reaction; Mazmanian et al., 2001). Even if the pentaglycine anchor structure of the cell wall is altered by incorporation of serine residues, anchoring to the cell wall is not affected (Strauss et al., 1998).

Staphylococcus aureus mutants lacking the *srtA* gene fail to anchor and display some surface proteins (mostly protein A and fibronectin-binding protein) and are impaired in the ability to cause animal infections (Mazmanian et al.,

Fig. 12. Cell wall sorting (cws) sequence of protein A.

Table 14. *Staphylococcus aureus* surface proteins.

Protein	Length (aa)	Ligand	References
Spa	508	IgG, vWBF	Uhlen et al., 1984
FnbA	1018	Fibronectin or fibrinogen	Signäs et al., 1989
FnbB	914	Fibronectin or fibrinogen	Jönsson et al., 1991
ClfA	933	Fibrinogen	McDevitt et al., 1994
ClfB	913	Fibrinogen	Ni Eidhin et al., 1998
Cna	1183	Collagen	Patti et al., 1994
SdrC	947	Unknown	Josefsson et al., 1998a
SdrD	1315	Calcium	Josefsson et al., 1998b
SdrE	1166	Bone sialoprotein	Tung et al., 2000
Pls	1637	None[a]	Savolainen et al., 2001
SasA to SasJ	ND	Unknown	Genome sequence

Abbreviation: aa, number of amino acids; vWBF, von Willebrand-Factor; Sas, *S. aureus* surface proteins; and ND, not determined.
[a]Inhibits adhesion to fibronectin or fibrinogen.

2000). Sortase acts on surface proteins that are normally secreted by the classical secretory "sec" pathway and whose signal peptide is removed by the signal peptidase.

Recently, a second sortase gene (*srtB*) was identified. SrtB appears to be required for anchoring of a surface protein with an asparagine-proline-glutamine-threonine-asparagine (NPQTN) motif. An *srtB* mutant is defective in the persistence of animal infections. The *srtB* gene is part of an iron-regulated locus called "iron-responsive surface determinants" (*isd*), which also contains a ferrichrome transporter and surface proteins with NPQTN and LPXTG motifs. It is suggested that SrtB might be involved in iron acquisition (Mazmanian et al., 2002).

When tethered to the C-terminus of other proteins bearing N-terminal signal peptides, the cws promotes anchoring of hybrid protein to the cell wall (Schneewind et al., 1993; Navarre et al., 1996). It was also possible to anchor enzymes such as lipases or β-lactamases in active form to the staphylococcal cell wall, provided that a cell wall spanning region is upstream of the LPXTG motif (Strauss and Götz, 1996).

Slime and Biofilm Formation

The molecular and biochemical basis of biofilm formation in *S. aureus* and *S. epidermidis* has been recently reviewed (Götz, 2002). The first reports that slime-producing *S. epidermidis* strains are involved in catheter-associated sepsis and infections came from the groups of Peters (Peters et al., 1981), Christensen (Christensen et al., 1982), and Costerton (Marrie et al., 1982b). Mostly *S. epidermidis*, but sometimes also mixed cultures have been isolated from colonized catheters recovered from patients. The bacteria appeared to be closely packed and cemented together by a slimy matrix (Peters et al., 1981). Transmission and scanning electron micrographs showed again that *S. epidermidis* forms a confluent biofilm, in which the cells are embedded in an amorphous material referred to as "slime" (Christensen et al., 1982). These early observations and pioneering work indicated that 1) the skin bacterium *S. epidermidis*, once considered to be harmless, is an opportunistic pathogen that could cause chronic staphylococcal infection and 2) in certain strains, slime (an extracellular polysaccharide) is produced and contributes to the formation of a confluent biofilm. As summarized recently, *S. epidermidis* can form a biofilm on nearly any synthetic polymers used as prosthetic devices, and in addition, they can bind to blood proteins, matrix proteins, and human cell receptors (Götz and Peters, 2000a; Götz et al., 2000b).

Today *S. epidermidis* is regarded as a leading species in causing chronic polymer-associated clinical infection. Microscopy of biofilm formation in vitro suggests that two steps are involved: 1) the attachment of the bacterial cells to the polymer surface, and 2) the growth-dependent accumulation to form multilayered cell clusters surrounded by a slimy matrix (Fig. 13).

The slime consists of a polysaccharide composed of β-1,6-linked *N*-acetylglucosamine, 15–20% of which is de-acetylated and therefore positively charged (Mack et al., 1996). Also identified was another polysaccharide II (<20%), which is structurally related, but has a lower content of non-*N*-acetylated D-glucosaminyl residues and contains phosphate and ester-linked succinate, rendering it anionic. The structure of this polysaccharide is unique, and according

2-step model of biofilm formation in staphylococci

1. Adhesion of bacterial cells to a surface
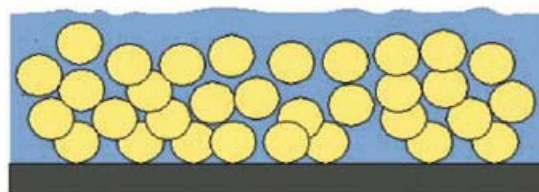
2. Proliferation of cells and embedding in a slimy matrix, mainly based on the polyglucosamine (PIA)
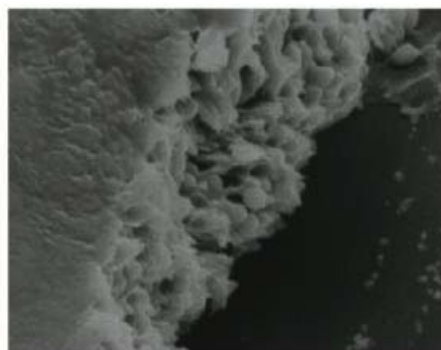
3. EM photograph of a *S. epidermidis* biofilm developed on plastic surface

Fig. 13. Two-step model of staphylococcal biofilm formation. The first step is the adherence of the bacterial cells to a surface. The second is the embedding of the cells in a thick slime matrix (biofilm). Within the biofilm, cells appear to have reduced physiological activity in an anoxic environment, and exhibit a decreased sensitivity to many antibiotics, compared with their planktonic counterparts. See also Götz and Peters (2000a).

to its function in intercellular aggregation, it was referred to as "*polysaccharide intercellular adhesin*" (PIA; Mack et al., 1996). A recently described polysaccharide PNSG (poly-*N*-succinyl β-1-6 glucosamine; McKenney et al., 1999) from *S. aureus* MN8N, containing *N*-acetylglucosamine residues regarded as completely succinylated, turned out to be an artefact (G. Pier, personal communication). PIA has several functions (Götz, 2002): it is a major factor of biofilm formation, contributes to the pathogenesis of biomaterial-associated infections (Rupp et al., 2001), is immunogenic, is involved in hemagglutination, binds to hydrophilic surfaces, and finally causes intercellular adhesion. Detection methods for PIA and biofilm formation have been recently described (Cramton et al., 2000; Leriche et al., 2000).

Polysaccharide Intercellular Adhesion Biosynthesis The genes responsible for biofilm formation were identified by isolating and analyzing biofilm-negative transposon-insertion mutants of *S. epidermidis* O-47 (Heilmann et al., 1996a). In this way, a number of genes could be identified which are involved in biofilm formation. There is the *ica* (*intercellular adhesion*) operon, which is responsible for the biosynthesis of PIA and its transcriptional control (Heilmann et al., 1996b). The operon is composed of the *icaR* (regulatory) gene and *icaADBC* (biosynthesis) genes. The in vitro biosynthesis of PIA has been analyzed using the membrane fraction or cellular or extracellular extracts (Gerke et al., 1998). IcaA has *N*-acetylglucosaminyl-transferase activity with UDP-*N*-acetyl-glucosamine as substrate. IcaD might be a chaperone that directs the correct folding and membrane insertion of IcaA and, in addition, might act as a link between IcaA and IcaC. The role of IcaC is still unclear.

Other biofilm-negative staphylococcal mutants were defective in adherence to polymers and extracellular matrix. The deletion of the major autolysin *atlE* gene revealed a pleiotropic effect (Heilmann et al., 1997): Because of their amidase deficiency, the mutant cells form huge cell clusters that resist even detergent treatment, which points to a defect in cell separation. Owing to extracellular processing of the precursor AtlE, five surface-bound proteins were missing. Binding to vitronectin and fibronectin was reduced, and finally, adherence to and biofilm-formation on hydrophobic surfaces was markedly decreased.

In a search for *S. aureus* mutants hypersensitive to positively charged antimicrobial peptides, mutants with an altered teichoic acid structure have been isolated (Peschel et al., 1999). The mutations are in the *dltA* operon, and the teichoic acids lack D-alanine. It has been postulated that the increased negative charge of the cell surface of the mutants leads to an increased scavenging of positively charged antimicrobial peptides, thus leading to the observed hypersensitivity. The *dltA* mutant is also biofilm-negative even though PIA production appears to be unchanged. The mutant is severely affected in adherence to polystyrene or glass surfaces (Gross et al., 2001), the first step of biofilm formation. In this respect, the *dltA* mutant resembles the *atlE* mutant described above, with one exception: the *atlE* mutant is still able to form a biofilm on a glass surface. That biofilm formation of the *dltA* mutant can be completely restored by the addition of Mg^{+2} ions corroborates the biological importance of the charge balance of the Gram-positive cell surface.

Other proteins can also contribute to biofilm formation in staphylococci, such as the accumulation-associated protein (AAP; Hussain et al., 1997) and the biofilm-associated protein (Bap; Cucarella et al., 2001). Especially *S. aureus* is capable of adhering to a large variety of matrix components to initiate colonization. This adherence is frequently mediated by protein adhesins of the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family, which in many cases are covalently anchored to the cell wall peptidoglycan (Foster and Höök, 1998). Very likely, surface proteins such as fibronectin-binding protein A and B (FnBPA and FnBPB), the collagen-binding protein Cna, and the fibrinogen-binding proteins, clumping factor A and B (ClfA and ClfB), contribute to adherence and thus to biofilm formation.

Slime production and biofilm formation are regulated by a number of environmental parameters and stress factors, which were summarized by Götz (2002). Among them, anaerobiosis clearly induces ica expression (Cramton et al., 2001). It was also shown that IS*256* integrates into the ica operon and can also be precisely excised (Ziebuhr et al., 1999; Ziebuhr et al., 2000a; Ziebuhr et al., 2000b). The role of PIA in pathogenicity is also corroborated by the finding that an intact *ica* operon is more prevalent in clinical (septicemic disease and shunt-associated meningitis) *S. epidermidis* isolates than in skin isolates of nonhospitalized persons (Ziebuhr et al., 1997).

Biofilm formation in staphylococci is multifactorial, and the ability to form a biofilm makes the strains much better able to survive in the normally hostile environment of tissue and blood. Biofilm formation appears to be a bacterial survival strategy that is turned on when, for example, oxygen and Fe ions become limited, when sublethal concentrations of certain antibiotics are present, or other stress

situations emerge. The modulation of biofilm formation by various environmental conditions appears to be an advantage for successful infection.

New Membrane Components Another mutant type was hypersensitive to CAMPs (Peschel et al., 2001) and identified with a novel staphylococcal gene, *mprF*. The *mprF* mutant strain was killed considerably faster by human neutrophils and exhibited attenuated virulence in mice, indicating a key role for defensin resistance in the pathogenicity of *S. aureus*. In the *mprF* mutant, the predominant membrane component phosphatidylglycerol lacks L-lysine modification. The structure of lysylphosphatidylglycerol (L-PG) is shown in Fig. 14. As this unusual modification leads to a reduced negative charge of the membrane surface, MprF-mediated peptide resistance is most likely based on repulsion of the cationic peptides. Accordingly, inactivation of *mprF* led to increased binding of antimicrobial peptides by the bacteria. The *mprF* gene has no similarity with genes of known function, but related genes were identified in the genomes of several pathogens including *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*. MprF thus constitutes a novel virulence factor, which may be of general relevance for bacterial pathogens and represents a new target for attacking multidrug resistant bacteria.

Protein Secretion in Staphylococci

The *S. aureus* genome was screened for secretion genes of the classical sec pathway (Kuroda et al., 2001; Mazmanian et al., 2001). Apart from the *E. coli* specific *secB* and *secF* genes, all other genes (*secA, D, E, G, Y*; *yajC*; *ffh*; *ftsY*, and *lepB*) are also present in the *S. aureus* genome. In addition, the *S. aureus* genomes revealed two *secA* (*secA*-1 and *secA*-2) and two *secY* genes (*secY*-1 and *secY*-2). The presence of two homologous genes is unclear; however, one can speculate that they play a role for a specific set of translocated proteins, like for example *srtB*, whose function differs apparently from that of *srtA*. The presence of most of the *sec* genes suggests that in *S. aureus*, the sec pathway is essentially similar to that described for *E. coli*. Maybe this is the reason why protein secretion is hardly studied in staphylococci. However, it was shown that many of the secreted proteins in staphylococci possess a so-called "pro-peptide region" and the role of this region in protein expression and secretion has been studied in more detail.

Role of the Pro-peptide Region in Exoproteins Since the *S. hyicus* specific lipase (SHL) was the first bacterial lipase gene ever to be cloned and sequenced (Götz et al., 1985), both the enzymatic properties and the role of the pro-peptide was investigated in more detail with this enzyme (reviewed in Götz and Rosenstein, 2001b).

According to sequence comparisons, all staphylococcal lipases are predicted to be primarily pre-pro-lipases. A structural organization of some of the lipases is shown in Fig. 15. The various pro-peptides have no striking similarities at the sequence level but are distinguished by
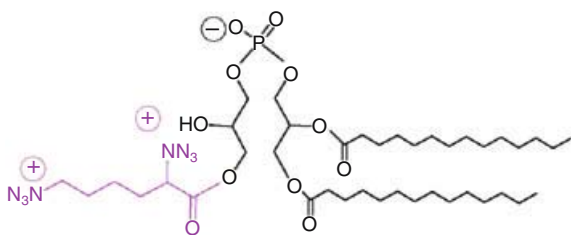
Fig. 14. Structure of lysylphosphatidylglycerol (L-PG).

Fig. 15. Organization of staphylococcal (phospho)lipases as pre-pro-enzymes. SHL, (phospho) lipase from *S. hyicus* subsp. *hyicus* DSM 20459; SAL-2, lipase from *S. aureus* NCTC 8530; SAL-1, lipase from S. aureus PS54; SEL-2, lipase from *S. epidermidis* RP62A. sp, signal peptide; and pp, pro-peptide region. The proposed active site amino acids are indicated in the mature form of each enzyme. aa, amino acid number or position; and IEP, isoelectric point. From Götz et al. (1998).
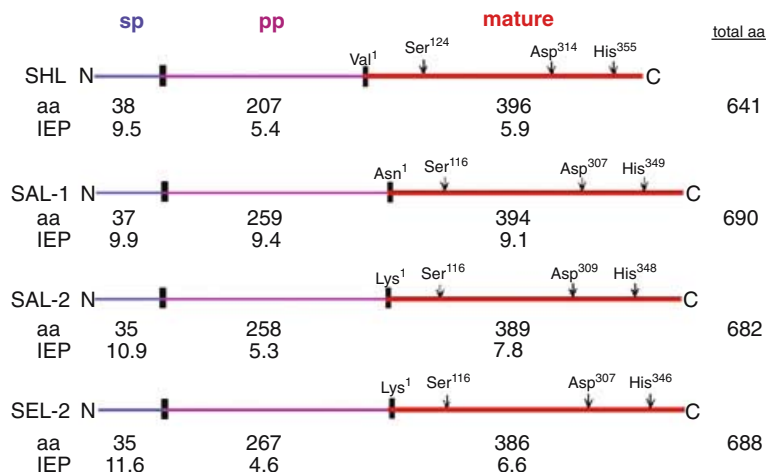
their overall hydrophilic character. To get more insight into the function of the pro-peptide, internal deletions within this region were made and analyzed (Demleitner and Götz, 1994). The results obtained with these lipase mutants indicate that the SHL pro-peptide may have two functional domains, with each one located in one half of the pro-region. The N-terminal part seems to be important for lipase activity and the C-terminal part, for translocation and stability. A stabilizing effect of the SHL pro-peptide has also been observed in an experiment where OmpA of *E. coli* is fused to the pre-pro portion of SHL; without the SHL pro-peptide, massive proteolytic degradation occurred after secretion by *S. carnosus* (Meens et al., 1997). A number of experiments designed to address the question whether the pro-peptide could act also in *trans* indicated that it has no *trans* activity. It exerts its beneficial effects on translocation, stability, and activity only intramolecularly (G. Thumm et al., unpublished observation). All studies carried with the pro-peptide speak in favor of an intramolecular chaperon-like function. Maybe it compensates in *Staphylococcus* and also in *Bacillus* for the lack of the SecB homolog.

The proteolytic processing of pro-SHL has been studied in more detail (Liebl and Götz, 1986; Götz, 1991; Demleitner and Götz, 1994). Since the 86-kDa pro-SHL purified from *S. carnosus* is processed by culture supernatants of *S. hyicus*, it became apparent that the processing of the pro-region by an extracellular protease occurs after secretion. The proteolytic activities in the supernatant of *S. hyicus* were analyzed, and two proteases (ShpI and ShpII) were identified; ShpII proved to be involved in pro-SHL processing (Ayora and Götz, 1994a; Ayora et al., 1994b).

THE USE OF SHL AS EXPRESSION AND SECRETION SYSTEM The beneficial role of the pro-peptide region of SHL for efficient secretion of heterologous proteins and enzymes was already described earlier (Liebl and Götz, 1986). The results support a dual role for the SHL pro-peptide: an involvement in protein translocation and a role in stabilization against proteolytic degradation.

Because of these encouraging results, the pre-pro-portion of SHL was fused to several other heterologous proteins such as human growth hormone (Sturmfels et al., 2001) and malaria antigen (Samuelson et al., 1995). In all cases, the heterologous proteins were successfully and in good quantities secreted by *S. carnosus*, indicating a general application of this strategy.

An alignment of the lipase signal peptides shows a remarkable motif containing the perfectly conserved residues Ser, Ile, Arg and Lys,

designated as the "SIRK-motif" (Götz et al., 1985; Nikoleit et al., 1995). This motif is present in many exoproteins of Gram-positive bacteria. Its function is still unclear.

Genomic Analysis of Virulence Genes

Several staphylococcal strains are completely sequenced but so far only the *S. aureus* strains N315 and Mu50 (Kuroda et al., 2001) are annotated and permit a general protein search. Some general features of *S. aureus* N315 genome are listed in Table 15.

To see whether and to which extent the genomes of *S. aureus* N315 (causing acute infections) and *S. epidermidis* RP62A (causing chronic infections) differ from each other, an arbitrary list of 125 virulence or fitness genes of the *S. aureus* N315 genome were investigated for the presence of homologous genes in *S. epidermidis* RP62A (Nerz et al., 2002). Genes or operons were selected that are involved in the production of exoenzyme, toxins, adhesins including biofilm formation, Fe-uptake, resistance to various antibiotics, and other functions. The comparison of both genomes was complicated by the fact that the RP62A sequence was

Table 15. General features of *Staphylococcus aureus* N315 genome.

Chromosome	
Length of sequence (bp)	2,813,641
G+C content (total genome)	32.8%
ORFs Percentage coding	84.5%
Protein coding regions	2595
Ribosomal RNAs	
16S	5
23S	5
5S	6
t-RNAs	62
tmRNAs	1
Insertion sequences	
IS*1181*	8
IS*431*	2
Others	10
Transposons	
Tn*554*	5
Others	0
Bacteriophages	1
SCCmec	1
Pathogenicity islands	**3**
Plasmid	
Length of sequence (bp)	2465

Abbreviations: tmRNA, RNA that has dual tRNA-like and mRNA-like nature; IS, insertion sequence; Tn, transposon; SCCmec, staphylococcal cassette chromosome mec; and mec, methicillin-resistance component.
From Kuroda et al. (2001).

not yet annotated. The selected virulence genes were localized on the *S. aureus* N315 genome (at the DOGAN-Server) by their ID-number and translated into amino acid sequence. The protein sequences were used as queries in a tBLASTn search of the *S. epidermidis* RP62A genome at The Institute for Genomic Research (TIGR) Web site. We also checked the almost finished genome sequence of the food-grade *Staphylococcus carnosus* TM300 (R. Rosenstein, C. Nerz, and F. Götz). To evaluate whether the resulting matches could be regarded as significant and thus represent homologous genes, they were checked carefully for length of alignment, percentage of similarity/identity and presence of functional protein domains. For this analysis, we used the following Web sites/tools:

In Tables 16 to 20, the genes identified in *S. aureus* N315 and the corresponding protein names (if available) are indicated. Those genes that have homologous counterparts in *S. carnosus* TM300 are highlighted in red.

Of the 159 selected virulence genes in *S. aureus* N315, only 37 genes (18%) had a homologous equivalent in *S. epidermidis* RP62A and 31 genes had homologous equivalent in *S. carnosus* TM300 (Rosenstein, Nerz, and Götz). This clearly reflects the difference in the pathogenic potential of the three species representatives and explains why *S. aureus* is rather an aggressive pathogen, *S. epidermidis* a mild pathogen and *S. carnosus* is non-pathogenic. Of the 40 toxin genes in *S. aureus*, only 3 and 5, respectively, were identified in the *S. epidermidis* and *S. carnosus* genome. This finding is in agreement with the earlier observations of decreased toxin production of *S. epidermidis* and corroborates well with the decreased severity of an *S. epidermidis*

Table 16. Presence of exoenzymes in *S. aureus* N315/Mu50 and *S. carnosus* TM300.

Exoenzymes	ORFs	Gene name	Function	Similarity (%)
Staphylocoagulase	SA0222	*coa*	Possible coagulation in tissues (agr-controlled)	—
Possible staphylocoagulase	SA0743	None	Unknown	—
Thermonase	SA1160	*nuc*	Degradation of host's nucleic acid	77
Staphylococcal nuclease Micrococcal nuclease[a]	SA0746	*nuc*	Degradation of host's nucleic acid (agr-controlled)	—
Probable 59-nucleotidase	SA0022	None	Unknown	62
Lipase	SA0309	*geh*	Hydrolytic degradation of lipids	—
Triacylglycerol lipase	SA2463	*lip*	Hydrolytic degradation of lipids	56
Similar to lipase LipA	SA0610	None	Unknown	77
Probable lipase	SA2323		Unknown	—
1-Phosphatidylinositol phosphodiesterase	SA0091	*plc*	Hydrolysis of phosphatidylinositol	—
Hyaluronate lyase[b]	SA2003	*hysA*	Degradation of host's hyaluronic acid	—
Serine protease[c]	SA0901	*sspA*	Proteolytic destruction of host tissues (agr-controlled)	—
Serine protease	SA0879	None	Proteolytic destruction of host	—
Probable serine protease	SA1627, 1628, 1629, 1630, and 1631 Location: SaPIn3/ SaPlm3	*splA*, *splB*, *splC*, *splD*, and *splF*	Unknown	—
Cysteine protease	SA0900	*sspB*	Proteolytic destruction of host tissues	—
Staphopain[d]	SA1725	None	Proteolytic destruction of host tissues	—
Zinc metalloproteinase aureolysin	SA2430	*aur*	Proteolysis of host tissues	—
Staphylokinase[e]	SA1758 Location: N315/ Mu50A	*sak*	Proteolytic destruction of host tissues	—

Symbol and abbreviations: −, not determined; ORFs, open reading frames; agr, accessory gene regulator; and Tnase, thermonuclease.

[a] EC-Number 3.1.31.1; typical for *S. aureus*.
[b] Hyaluronidase.
[c] V8 protease.
[d] Cysteine proteinase.
[e] Protease III.
Blue indicates newly identified ORFs.
Red indicates counterpart in the *S. carnosus* TM300 genome.

Table 17. Presence of toxins in *S. aureus* N315/Mu50 and *S. carnosus* TM300.

Toxins	ORFs	Gene name	Function	Similarity (%)
α-Hemolysin	SA1007	*hla*	Destruction of blood and tissue cells (agr-controlled)	—
γ-Hemolysin components	SA2207, 2208, and 2209	*hlgA*, *hlgC*, and *hlgB*	Destruction of blood cells	—
δ-Hemolysin	SAS065	*hld*	Destruction of blood and tissue cells (agr-controlled)	77
Leukotoxins	SA1637 and 1638 Location: SaPIn3/SaPIm3	*lukD* and *lukE*	Destruction of white blood cells	—
Possible leukocidin	SA1813	*lukM*	Unknown	—
Possible hemolysin	SA0657	None	Unknown	89
Possible hemolysin	SA0780	None	Unknown	82
Similar to exotoxin 1	SA1009	None	Unknown	55
Possible hemolysin	SA1812	None	Unknown	—
Similar to hemolysin III	SA1973	None	Unknown	75
Possible exotoxins	SA0357	None	Unknown	—
	SA0382, 0383, 0384 Location: SaPIn2/SaPIm2	*set6*, *set7*, *set8*, *set9*	Unknown	—
	0385, 0386, 0387	*set10*, *set11*, *set12*		—
	0388, 0389, 0390, and 0393	*set13*, *set14*		—
	SA1009, 1010, and 1011		Unknown	—
Superantigens Enterotoxins	SA1642, 1643, 1644, 1645, 1646, 1647, and 1648	*seg*, *sen*, *ent2*, *ent1*, *sei*, *sem*, and *seo*	Food poisoning, superantigen (agr-controlled)	—
Enterotoxin P	SA1761(N315)	*sep*	Food poisoning, superantigen	—
Enterotoxin A	SAV119(Mu50)	*sea*	Food poisoning, superantigen	—
Enterotoxins	SA1816 and 1817 Location: SaPIn1/SaPIm1	*sel* and *sec3*	Food poisoning, superantigen	—
Toxic shock syndrome toxin 1	SA1819 Location: SaPIn1/SaPIm1	*tst*	Fever, shock, skin rash, superantigen (agr-controlled)	—
Probable enterotoxin	SA1430	None	Unknown	—

Symbol and abbreviation: –, not determined; and agr, accessory gene regulator.
Blue indicates newly identified ORFs.
Red indicates counterpart in the *S. carnosus* TM300 genome.

Table 18. Presence of adhesins in *S. aureus* N315/Mu50 and *S. carnosus* TM300.

Adhesins	ORFs	Gene name	Function	Similarity (%)
IgG-binding protein A	SA0107	*spa*	Potential immune disorder in host (agr-controlled)	—
IgG-binding protein SBI	SA2206	*sbi*	Potential immune disorder in host	—
Ser-Asp-rich fibrinogen-binding proteins	SA0742 and 2423	*clfA, clfB*	Cellular adhesion onto host tissues	—
Possible fibrinogen-binding proteins	SA1000, 1003, and 1004	None	Unknown	—
Fibronectin-binding proteins	SA2290 and 2291	*fnbB* and *fnbA*	Cellular adhesion onto host tissues (agr-controlled)	—
Ser-Asp-rich proteins	0519, 0520 and 0521	*sdrC*, *sdrD* and *sdrE*	Cellular adhesion onto host tissues	—
Possible extracellular matrix binding proteins	SA0744 and 0745		Cellular adhesion onto host tissues	—
Probable extracellular matrix binding proteins	SA1267 and 1268	*ebhA* and *ebhB*	Unknown	—
Sortase A	SA2316	*srtA*	Covalent linkage of binding proteins to the peptidoglycan	74[a]
Sortase B	SA0982	*srtB*	Covalent linkage of binding Fe-protein to the peptidoglycan	—

Table 18. *Continued*

Adhesins	ORFs	Gene name	Function	Similarity (%)
Proteins with the cell wall sorting signals (LPXTG)	SA0976, 0977, 1552, 1577, 1888, 1964, 2284, 2381, and 2447	None	Possible pathogenic factors	—
Probable adhesin	SA0587	None	Cellular adhesion onto host tissues	83
Elastin-binding protein	SA1312	*ebpS*	Cellular adhesion onto host tissues	—

Symbol and abbreviations: –, not determined; IgG, immunoglobulin G; and LPXTG, leucine-proline-unknown-threonine-glycine.
[a]Similarity: 77% (ubiquitous).
Blue indicates newly identified ORFs.
Red indicates counterpart in the *S. carnosus* TM300 genome.

Table 19. Presence of Fe-uptake and other virulence genes in *S. aureus* N315/Mu50 and *S. carnosus* TM300.

Fe-uptake	ORFs	Gene name	Function	Similarity (%)
Possible siderophore biosynthesis proteins	SA0116 and 0117	None	Iron uptake	—
Possible iron-binding protein	SA0217	None	Iron uptake	—
Possible iron permease components	SA0566 and 0567	None	Iron uptake	73, 60
Possible ferrichrome ABC transporter	SA0891	None	Iron uptake	60
Possible ferrichrome ABC transporter components	SA0980, 0981	None	Iron uptake	—
Possible ferritin	SA1709	None	Maintenance of cellular iron	—
Possible ferrichrome ABC transporter components	SA1977, 1978, and 1979	None	Iron uptake	77, 74, and 71
Ferrichrome ABC transporter FhuD homolog	SA2079	*fhuD*	Iron uptake	84
Possible iron transport proteins	SA2337 and 2369		Iron uptake	80, 70
Ferrichrome ABC transporter FhuD homolog	SAV0812	SaGIm[a]	Iron uptake	—

Others	ORFs	Gene name	Function	Similarity
Capsular polysaccharide synthesis proteins	SA0144-159	*cap A-P*	Possible escape from immune system	72–89 cap A-D, M,P
Probable capsular polysaccharide synthesis proteins	SA0126, 0127		Unknown	—
Intercellular adhesion proteins	SA2459, 2460, 2461, and 2462	*icaA*, *D*, *B* and *C*	Cell-cell aggregation on infected tissues	—
MHC class II-like protein Map-w	SA0841, 1750–51, 2006		Unknown	—
Myosin-crossreactive MHC class II-like protein	SA0102		Potential immune disorder in host	—
Synthesis of lysyl-phosphatidyl glyceride	SA1193	*mprF* (*fmtC*)	Resistance to cationic antimicrobial peptides[b]	75
D-Alanyl esterification of teichoic acids	SA0793-96	*dltA*, *B*, *C* and *D*	Resistance to cationic antimicrobial peptides[b]	89, 84, 99, and 84

Symbol and abbreviations: –, not determined; ORFs, open reading frames; ABC, ATP-binding cassette; and MHC, major histocompatibility complex.
[a]SaGIm is the genomic island name.
[b]Gene is widespread.
Blue indicates newly identified ORFs.
Red indicates counterpart in the *S. carnosus* TM300 genome.

Table 20. Presence of antibiotic resistance genes in *S. aureus* N315/Mu50 and *S. carnosus* TM300.

Resistance genes	ORF	Gene name	Function	Similarity (%)
Penicillin binding protein 29	SA0038	*mecA*	Pc resistance	—
β-Lactamase	SAP010	*blaZ*	Pc resistance[a]	—
rRNA adenine *N*-6-methyltransferase	SA0048	*ermA*	Em resistance[b]	—
rRNA adenine *N*-6-methyltransferase	SA0766	*ermA*	Em resistance	—
rRNA adenine *N*-6-methyltransferase	SA1480	*ermA*	Em resistance	—
rRNA adenine *N*-6-methyltransferase	SA1951	*ermA*	Em resistance	—
rRNA adenine *N*-6-methyltransferase	SA2348	*ermA*	Em resistance	—
O-nucleotidyltransferase	SA0049	*ant*	Ags resistance[b]	—
O-nucleotidyltransferase	SA0765	*ant*	Ags resistance	—
O-nucleotidyltransferase	SA1481	*ant*	Ags resistance	—
O-nucleotidyltransferase	SA1952	*ant*	Ags resistance	—
O-nucleotidyltransferase	SA2385	*ant*	Ags resistance	—
Bifunctional AAC/APH protein	SAVP026	*aacA*	Ags resistance	—
Tetracycline resistance protein	SAV0387	*tetM*	Tc resistance	—
Bleomycin resistance protein	SA0032	*bleO*	Ble resistance	—

Symbol and abbreviations: −, not determined; ORF, open reading frame; Pc, penicillin; Em, erythromycin; Ags, aminoglycoside; Tc, tetracycline; Ble, bleomycin; and AAC/APH, 6′-aminoglycoside-*N*-acetyltransferase and 2′-aminoglycoside phosphotransferase.

[a]Two copies.

[b]Three copies.

infection. Compared to *S. aureus*, *S. carnosus* was also found to have a much decreased number of other potential virulence factors such as exoenzymes and adhesins.

This is only a preliminary study since the *S. epidermidis* and *S. carnosus* genomes were only screened for the presence of known *S. aureus* virulence genes. On the other hand, not many virulence genes are known in *S. epidermidis*. However, this first analysis may shed some light why *S. epidermidis* causes rather mild or chronic infections. In the future, we shall get a more complete picture if the genomes of other staphylococcal species are included in the comparative genome analysis.

The Regulatory Network in Staphylococci

Accessory Gene Regulation

The initial step in an infectious disease is often adhesion to and colonization of host tissue surfaces. *Staphylococcus aureus* has been shown to bind to several host matrix proteins and plasma proteins, such as fibronectin (Flock et al., 1987; Froman et al., 1987; Jonsson et al., 1991), fibrinogen (Boden and Flock, 1989), collagen (Foster and Höök, 1998), elastin (Park et al., 1991), laminin (Vercellotti et al., 1985; Herrmann et al., 1988), prothrombin (Kawabata et al., 1985), thrombospondin (Herrmann et al., 1991), bone sialoprotein (Ryden et al., 1990; Yacoub et al., 1994), and vitronectin (Liang et al., 1995; Heilmann et al., 1997). For each of these binding

functions, a corresponding surface-associated protein has been identified. The existence of an *S. aureus* extracellular matrix binding protein with broad specificity that is capable of binding several extracellular glycoproteins has also been reported (McGavin et al., 1993). The role of some of these proteins in the pathogenesis of staphylococcal infections has been shown in animal models (Foster and McDevitt, 1994; Moreillon et al., 1995; Flock et al., 1996; Palma et al., 1996).

The expression of many matrix-binding proteins is controlled by the agr (accessory gene regulator) system. Many of the genes coding for extracellular toxins, enzymes, and cell surface proteins in *S. aureus* are regulated by a 510-nucleotide RNA molecule, RNAIII. Transcription of genes encoding secreted toxins and enzymes (including *hla* [α-toxin], *saeB* [enterotoxin B], *tst* [toxic shock syndrome toxin 1], and *ssp* [serine protease]) is stimulated, while transcription of genes encoding cell surface proteins (like *spa* [protein A] and *fnb* [fibronectin binding proteins]), is repressed (Tegmark et al., 1998).

In *S. aureus*, transcription of at least 15 virulence genes, encoding extracellular toxins, enzymes, and cell surface proteins, is also regulated (Janzon and Arvidson, 1990; Novick et al., 1993). Production of toxins and enzymes is generally positively controlled, while that of cell surface proteins is negatively controlled (Janzon et al., 1989; Kornblum et al., 1990). In Table 21, agr-controlled genes are classified according to their degree of regulation (Kornblum et al., 1990). For example, the expression of α-hemolysin, serine protease, toxic shock syndrome toxin (TSST-1), and δ-lysin is enhanced in an agr⁺ and very low

Table 21. Classification of agr-regulated extracellular protein expression in *Staphylococcus aureus*.

Class	Exoprotein	Gene	agr⁺	agr⁻
I	α-hemolysine	*hla*	high	undetectable
	serine protease	*spr* (V8)	high	undetectable
	TSST-1	*tst*	high	undetectable
	δ-hemolysin	*hld*	high	undetectable
II	nuclease	*nuc*	high	low-moderate
	β-hemolysin	*hlb*	high	low-moderate
	Enterotoxin B	*entB*	high	low-moderate
III	Protein A	*spa*	low	high
	Coagulase	*coa*	low	high
	FibronectinBP	*fbn*	low	high
IV	β-Lactamase	*bla*	no effect	
	Enterotoxin A	*entA*	no effect	

in an agr⁻ background, while the expression of the cell wall-bound proteins such as protein A, coagulase, and fibronectin BP is downregulated.

Synthesis of RNAIII is induced when the concentration of an autocrine octapeptide in the environment has reached a certain level (Ji et al., 1995; Ji et al., 1997). Generally, this happens during the late exponential phase of growth in laboratory cultures, which means that cell surface proteins are produced during the early exponential phase, while secreted toxins and enzymes are produced mainly during the post-exponential phase of growth (Björklind and Arvidson, 1980; Vandenesch et al., 1993; Lebeau et al., 1994).

The *agr* locus contains two divergent transcription units, RNAII and RNAIII, driven by the promoters P2 and P3, both of which are active only from the mid-exponential phase of growth (Novick et al., 1995). RNAII contains four genes: *agrA*, *agrB*, *agrC* and *agrD*. The *agrC* and *agrA* genes code for the components of a classical two-component signal transduction system, where AgrC is the membrane-bound signal receptor and AgrA is the response regulator, which is required for transcription of the RNAIII molecule and the *agr* operon itself (Novick et al., 1993; Novick et al., 1995).

The autoinducing peptide (AIP) is encoded in and derived from the AgrD propeptide that is in an unknown way post-translationally processed, modified and secreted. Very likely AgrB (Ji et al., 1995; Ji et al., 1997), an integral membrane protein, is involved besides additional factors (Saenz et al., 2000). The resultant mature AIP is the ligand that binds to and activates the phosphorylation of AgrC (Lina et al., 1998), which in turn is thought to phosphorylate AgrA, and phosphorylated AgrA found to induce RNAII and RNAIII synthesis (Ji et al., 1995). RNAIII causes an upregulation of secreted virulence factors as well as the downregulation of surface proteins. The gene organization of the agr system is shown in Fig. 16.
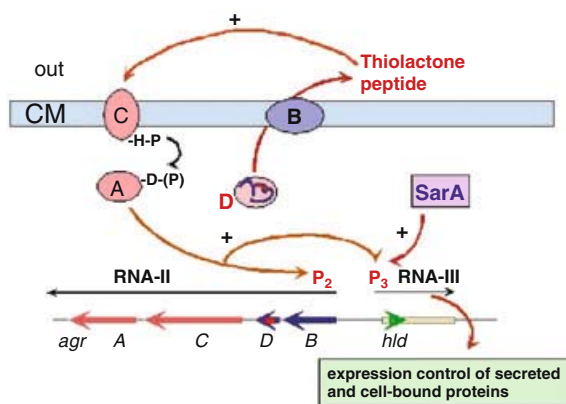
Fig. 16. Accessory gene regulatory (agr) genes and regulation cascade. From promoter region (P2), the RNA-II is transcribed encoding the genes *agrB*, *agrD*, *agrD*, *agrC* and *agrA*. The thiolactone peptide acts as an autoinducer that is sensed by the two component proteins AgrC and AgrA; the latter in its phosphorylated state activates transcription from P2 and P3. RNA-III controls gene expression of secreted and cell-bound proteins in an essentially unknown manner. RNA-III transcription is also positively controlled by the staphylococcal accessory regulator (SarA).

Quorum Sensing

The synthesis of RNAIII is regulated by a quorum sensing mechanism. The AIP molecules accumulate in the culture supernatant, and when a certain threshold concentration is reached, they interact with AgrC and induce as a consequence both RNAII and RNAIII transcription. Since transcription of P2 requires P2 operon products, the P2 operon is autocatalytic, and is thus suited for rapid production of secreted proteins at a time when overall growth is coming to a halt.

RNA III is the actual effector of the agr response, and incidentally encodes the agr-regulated peptide δ-lysin (Janzon et al., 1989), which is a 26-amino acid polypeptide that can form pores in membranes and lyse erythrocytes

(Freer and Birkbeck, 1982) but is not required for the regulation of target genes by RNAIII. How RNAIII interacts with target gene promoters is unknown.

Distribution of the Agr Locus

The *agr* locus, organized in the same way as that of *S. aureus*, has been demonstrated in the coagulase-negative *S. lugdunensis* (Vandenesch et al., 1993). However, RNAIII from *S. lugdunensis* does not code for δ-lysin, and its role in gene regulation is not known. The *agr* genes are also present in other coagulase-negative staphylococci such as *S. epidermidis*, *S. simulans* and *S. warneri* where RNAIII and δ-lysin homologues have been identified (Otto et al., 1998; Tegmark et al., 1998). In all RNAIII molecules, the first 50 and last 150 nucleotides were highly conserved, suggesting that these regions are important for the regulatory function.

Agr-specific Peptide Pheromones are Thiolactones

The agr-specific peptide pheromones are referred in the literature as AIP. However, the latter designation, does not consider the findings that these peptide pheromones not only induce the native agr system but also act at the same time as inhibitors of foreign agr systems. Therefore, the less restrictive term "peptide pheromone" is preferred.

The first hint that a peptide acts as an autoinducer of the agr system in *S. aureus* comes from the group of R. Novick. It turned out that the staphylococcal peptide pheromone sequences that are encoded in *agrD* gene vary from species to species. Even within *S. aureus*, four subtypes were identified (Ji et al., 1997). The first structure of a pheromone peptide and its biological activity was described in 1998 (Otto et al., 1998). The peptide sequence, DSVCASYF, was derived from AgrD of *S. epidermidis*. This synthetic octapeptide revealed only autoinducing activity when it contained the thiolactone ring between the central cysteine and the C-terminal carboxyl group (DSVc[CASYF]). The structure of the peptide pheromone of *S. epidermidis* is shown in Fig. 17. Unmodified peptides showed no activity (Otto et al., 1998). The receptor for the peptide pheromones, AgrC, must be very sensitive since the thiolactones are active at nanomolar concentrations. The N-terminus of the peptide pheromones is crucial for biological activity and specificity (Otto et al., 1998).

Interestingly, the *S. epidermidis* specific peptide pheromone is a potent inhibitor of the *S. aureus* agr system (Otto et al., 1999). However, while the correct N-terminus and the thiolactone
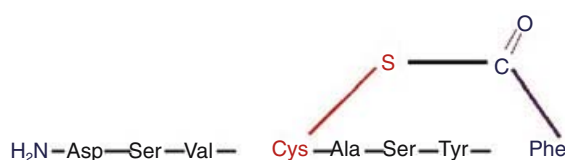
Fig. 17. Structure of agr pheromone (autoinducing peptide, AIP) of *S. epidermidis*. The agr pheromones are distinguished by a thiolester linkage between the central cysteine and the C-terminal carboxyl group. From Otto et al. (1998).

structure were absolute prerequisites for an agr-activating effect in *S. epidermidis*, inhibition of the *S. aureus* agr system showed a lesser structural stringency. Another important finding was that the corresponding lactone- and lactam-containing peptides DSVc[SASYF] and DSVc[DprASYF] inhibit the *S. aureus* agr system, but have lost autoinducing activity in its native *S. epidermidis* agr system (Otto et al., 1999). The *S. epidermidis* thiolactone inhibits the agr subgroups 1–3 but not 4 (Otto et al., 2001). An explanation for this could be that subgroup 4 thiolactone is very similar to that of *S. epidermidis*. A series of autoinducing peptide analogues (including the L-alanine and D-amino acid scanned peptides) were synthesized to determine the functionally critical residues within the *S. aureus* group I. It was found that 1) the group I autoinducing peptide (YSTc[CDFIM]) is inactivated in culture supernatants by the formation of the corresponding methionyl sulfoxide, and 2) that the lactam analogue retains the capacity to activate agr, suggesting that the thiolactone structure, which would allow covalent modification of the AgrC receptor, is not a necessary prerequisite for agr activation. Replacement of the endocyclic amino acid residue (D-aspartate) with alanine converted the group I thiolactone from an activator to a potent inhibitor. The addition of exogenous agr inhibitors to *S. aureus* decreased the production of TSST-1 and enterotoxin C3, confirming the potential of quorum-sensing blockade as a therapeutic strategy (Dowell et al., 2001).

It is very likely that in nature, there is a fierce competition between *S. aureus* and *S. epidermidis* and it looks like quorum-sensing cross talk generally favors survival of *S. epidermidis*, which might explain the predominance of *S. epidermidis* on the skin and in infections on indwelling medical devices (Götz, 2002).

Agr and Pathogenicity

In *agr* mutants, decrease in pathogenicity is very likely due to decreased synthesis of extracellular toxins and enzymes, such as α-, β-, and

δ-hemolysins, leucocidin, lipase, hyaluronate lyase, and proteases. In a murine arthritis model, the virulence was investigated of *S. aureus* 8325-4 and two *agr*/*hld* mutants derived from it (Abdelnour et al., 1993). Sixty percent of the mice injected with the wildtype strain developed arthritis, whereas *agrA* and *hld* mutants displayed joint involvement in only 10 and 30%, respectively. The question is also whether agr inhibiting thiolactone peptides can be therapeutically used. First tests showed that agr inhibitors exhibited biological activity in vivo in a mouse protection test (Mayville et al., 1999).

Staphylococcal Accessory Regulator

In *S. aureus*, the production of virulence factors such as cell wall adhesins and exotoxins during the growth cycle has been shown to be under the control of at least two regulator genes, *agr* and staphylococcal accessory regulator (*sar*; Manna et al., 1998). The global agr regulatory system is itself controlled by another regulator, namely the sar regulation system. This regulation system was first identified by Ambrose Cheung and his colleagues (Cheung et al., 1992). The sar locus consists of the 372-bp *sarA* preceded by a triple promoter region interspersed with two smaller open reading frames (ORFs) ORF3 and ORF4 (Bayer et al., 1996). The triple promoter system yields three overlapping sar transcripts, *sarA*, *sarC* and *sarB* of 0.56, 0.8 and 1.2 kb, respectively (Fig. 18). Expression studies with a *sigB* mutant revealed that the P3 promoter is SigB dependent (Manna et al., 1998). The protein sequence of the 14-kDa SarA shares sequence similarity with VirF of *Shigella flexneri* (Cheung and Projan, 1994).

Mutations in *sarA* resulted in a decreased expression of several extracellular (especially α-hemolysin) and cell wall proteins. This can be explained by the fact that transcription of RNAII and RNAIII of the agr system is also decreased.
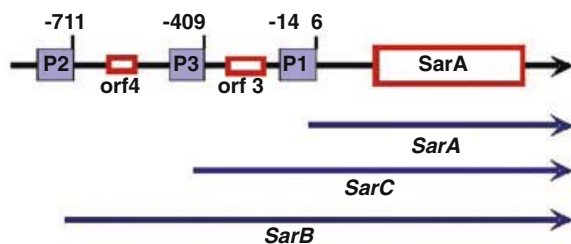
Fig. 18. Staphylococcal accessory regulator (sar) locus. The sar locus consists of the 372-bp *sarA* preceded by a triple promoter region (P1-P3) interspersed with two smaller open reading frames (ORF3 and ORF4; Bayer et al., 1996). The triple promoter system yields three overlapping sar transcripts, *sarA*, *sarC* and *sarB* of 0.56, 0.8 and 1.2 kb, respectively.

The *sarA* mutants can be complemented in part by plasmids expressing SarA, however, a full complementation was only achieved if *sarB* or *sarC* is expressed. These data suggest that ORF3, and to a lesser degree ORF4, may affect *agr* expression by modulating SarA protein expression. SarA binds to the P2 promoter region of *agr*, thus leading to transcription of RNAII and, subsequently, RNAIII, the two major transcripts encoded within the *agr* locus (Chien et al., 1998). The reduction in SarA expression correlated with a lower level of *agr* activation in the corresponding *sar* mutant clone.

However, in addition to controlling target gene expression via agr (e.g., α-hemolysin), the sar locus can also regulate target gene transcription via agr-independent mechanisms. In particular, it was found that SarA binds to conserved sequences homologous to the SarA-binding site on the agr promoter, upstream of the –35 promoter boxes of several target genes including *hla* (α-hemolysin gene), *spa* (protein A gene), *fnb* (fibronectin-binding protein genes), and *sec* (enterotoxin C gene; Chien et al., 1999). Deletion of the SarA recognition motif in the promoter regions of *agr* and *hla* in shuttle plasmids rendered the transcription of these genes undetectable in *agr* and hla mutants, respectively. Likewise, the transcription activity of *spa* (a gene normally repressed by *sar*) became derepressed in a wildtype strain containing a shuttle plasmid in which the SarA recognition site had been deleted from the *spa* promoter region. It is assumed that SarA is a regulatory protein that binds to its consensus recognition motif to activate (e.g., *hla*) or repress (e.g., *spa*) the transcription of *sar* target genes, thus accounting for both *agr*-dependent and *agr*-independent mode of regulation (Chien et al., 1999).

With a number of selected promoter regions, the one under control of SarA was investigated. Of the seven σ(A)-dependent promoters that were tested, SarA repressed transcription from agrP2, agrP3, *cna*, *sarP1*, and *sea* promoters and did not affect *sec* and *znt* promoters. Furthermore, SarA had no effect on transcription from the σ(B)-dependent *sar*P3 promoter. In vitro experimental data presented in this report suggest that SarA expression is autoregulated (Chakrabarti and Misra, 2000). SarA binds to conserved DNA motifs immediately upstream of both positively and negatively regulated promoters.

SarA interacts with a series of heptad repeats (AGTTAAG) within the agr promoter. Subsequent DNA-binding studies revealed that SarA binds readily to multiple AT-rich sequences of variable lengths. Crystal structure analysis of SarA and a SarA-DNA complex brought some insight to bear on the conformational changes

resulting from SarA encasement of DNA. SarA recognizes an AT-rich site in which the DNA is highly overwound and adopts a D-DNA-like conformation by indirect readout (Schumacher et al., 2001).

SarA Protein Family

The staphylococcal genome sequences revealed a number of SarA homologs. In search of additional regulators that could explain the differential effects of RNAIII and SarA, four differently regulated genes (*hla*, α-toxin; *hld*, RNAIII; *spa*, protein A; and *ssp*, serine protease) were analyzed for binding of potential regulatory proteins to the corresponding promoter DNA fragments linked to magnetic beads. One protein (29 kDa), with affinity for all four promoters, showed a high degree of similarity to SarA and was named "SarH1" (Sar homolog 1). Expression of *sarH1* was strongly repressed by *sarA* and *agr*. Further analysis revealed that *sarH1* has a strong repressive effect on *hla* and an activating effect on *spa* transcription (Tegmark et al., 2000). Furthermore, the production of several other exoproteins was affected by *sarH1*.

The level of SarA is partially controlled by the differential activation of sar promoters. With a DNA-specific column containing a sar P2 promoter fragment, a protein was purified which is encoded by *sarR*. SarR is a 13.6-kDa protein with homology to SarA. SarR binds to sar P1, P2, and P3 promoter fragments and a *sarR* mutant expressed a higher level of P1 transcript than the parent. As the P1 transcript is the predominant sar transcript, it was proposed that SarR is a sarA repressor protein that binds to the sar promoters to downregulate P1 transcription and, in consequence, SarA expression (Manna and Cheung, 2001).

The genome sequence revealed another SarA homolog, the 250-amino-acid (aa) SarS. Its gene is upstream of the *spa* gene. The expression of *sarS* was almost undetectable in parental *S. aureus* strain but was highly expressed in agr and *sarA* mutants, strains normally expressing a high level of protein A. Interestingly, protein A expression was decreased in a *sarS* mutant. The enhancement in *spa* expression in an *agr* mutant returned to a near-parental level in the *agr-sarS* double mutant, but not in the *sarA-sarS* double mutant. All the data indicated that *agr* probably mediates *spa* repression by suppressing the transcription of *sarS*, an activator of *spa* expression. However, the pathway by which the *sarA* downregulates *spa* expression is *sarS* independent (Cheung et al., 2001).

Another SarA homolog is the 118-aa long and basic SarT. Expression of *sarT* is repressed by *sarA* and *agr*. In a *sarT* mutant, the RNAIII level was notably increased particularly in the postex-

ponential phase. SarT repressed the expression of *hla* (α-hemolysin) similar to the repression in *agr* and *sarA* mutants. This finding suggests that *sarA*, contrary to the regulatory action of *agr*, induced α-hemolysin production by repressing *sarT*, a repressor of *hla* transcription (Schmidt et al., 2001).

Many of the SarA protein family members are either small basic proteins (<153 residues) or two-domain proteins in which a single domain has sequence similarity with one of the small basic proteins. Because of its structure and unique mode of DNA binding, SarR, and possibly other SarA family members, may belong to a new functional class of the winged-helix family, accommodating a long stretch of DNA with bending points. On the basis of sequence homology, it is hypothesized that the SarA protein family may entail homologous structures with similar DNA-binding motifs but divergent activation domains. An understanding of how these regulators interact with each other in vivo and how they sense environmental signals to control virulence gene expression (e.g., α-hemolysin) will provide more insight in the complex regulatory network (Cheung and Zhang, 2002).

Exoprotein Gene Regulator

In addition to *agr* and *sar*, another exoprotein gene regulator (*sae*) is involved in the expression of extracellular and cell surface proteins. In *sae* mutants, the production of α- and β-hemolysins and coagulase are drastically diminished and to a lesser extent also protein A. The *sae* locus regulates these exoprotein genes at transcriptional level (Giraudo et al., 1997). The *sae* mutation does not affect the expression of *agr* or *sar*. A *sae-agr* double mutant expressed reduced or null levels of α-, β-, and δ-hemolysins, coagulase, and high levels of protein A. The corresponding genes are not transcribed, while *spa* is transcribed at high levels. It is assumed that *sae* and *agr* interact in a complex way in the control of the expression of the genes of several exoproteins. The *sae* locus consists of two genes, designated "*saeR*" and "*saeS*," encoding a typical response regulator and histidine protein kinase, respectively (Giraudo et al., 1999).

In a guinea pig model of device-related infection, the impact of *agr*, *sarA* and *sae* mutants on the induction of *hla* (α-toxin gene) transcription was studied (Goerke et al., 2001). *Staphylococcus aureus* strains RN6390 and Newman expressed considerably smaller amounts of RNAIII in the guinea pig than during in vitro growth. Highest RNAIII and *hla* expression was detected in both strains early in infection, decreased during the course of infection that suggests that it was negatively correlated with

bacterial densities. The *agr* and *sarA* mutants of strains Newman and RN6390 did not affect *hla* expression in vivo, while in *sae* mutants, *hla* is severely downregulated in vitro as well as in vivo. This study suggests that *S. aureus* seems to be provided with regulatory circuits different from those characterized in vitro to ensure α-toxin synthesis during infections (Goerke et al., 2001).

Repressor of Toxins

Recently a gene called "repressor of toxins" (*rot*) was identified in *S. aureus* RN6390 that shows homology with *agrA* and *sarA* (McNamara et al., 2000). In a *rot-agr* double mutant the expression of protease and α-toxin is restored. This phenotype suggests that Rot acts as a transcriptional repressor of *hla*. Whether other genes are controlled by Rot is unknown.

The *arlS-arlR* Two-component System

In a *S. aureus* 8325 derivative, another two-component system, *arlS-arlR*, was identified which is involved in regulation of exoproteins (Fournier and Hooper, 2000a). ArlS is the sensor protein while ArlR represents the response regulator. An *arlS* mutant showed pleiotropic effects. It formed a biofilm on a polystyrene surface unlike the parent strain and the complemented mutant strain. Biofilm formation was associated with increased primary adherence to polystyrene, whereas cellular adhesion was only slightly decreased. In addition, the *arlS* mutant exhibited increased autolysis and altered peptidoglycan hydrolase activity. Another effect of the *arlS* mutant is that the activity of multidrug resistance efflux pump, NorA, is increased (Fournier et al., 2000b). While in the *arlS* or *arlR* mutants, the extracellular proteolytic activity, including serine protease activity, is dramatically decreased (Fournier and Hooper, 2000a), and transcription and production of other secreted proteins such as α- and β-hemolysins, lipase, coagulase, or the cell-wall bound protein A are increased (Fournier et al., 2001). The *arl* operon decreases the production of virulence factors by transcriptional downregulation of the corresponding genes. Since the *arl* mutation did not change *spa* expression in an *agr* or *sarA* mutant, it is suggested that arl acts indirectly on virulence gene expression through *agr* and *sar*. This is supported by the finding that RNAII and RNAIII are decreased by ArlS-ArlR, while *sarA* expression is increased.

Staphylococcal Virulence Regulator

The staphylococcal virulence regulator gene *svrA* was originally identified in *S. aureus* by signature-tagged mutagenesis as necessary for virulence. SvrA is a membrane-associated protein, having two regions with six membrane-spanning domains, separated by an extended hydrophilic loop (Garvis et al., 2002). The *svrA* mutant expressed greatly reduced amounts of α-, β- and δ-toxins and an increased amount of protein A; the regulation occurred at the transcriptional level. As the agr-specific RNAII and RNAIII were absent in the *svrA* mutant, it is assumed that SvrA is, like many other regulators, required for expression of agr.

The SrrA-SrrB Two-component System

It was found that the expression of toxic shock syndrome toxin 1 (TSST-1) by *S. aureus* in liquid culture consumes oxygen. In the course of studies examining the mechanism by which oxygen might regulate toxin production, the *srrAB* (staphylococcal respiratory response) genes were identified. The genes are homologous to the *B. subtilis resDE* genes. ResD-ResE represents a global regulator of aerobic and anaerobic respiratory metabolism in *B. subtilis*. The two-component regulatory system SrrA-SrrB very likely acts in anaerobic repression of staphylococcal virulence factors (Yarwood et al., 2001). In *srrB* mutants, RNAIII synthesis was upregulated while exotoxin TSST-1 synthesis was downregulated under microaerobic conditions and, to a lesser extent, under aerobic conditions as well. At the same time, protein A production was upregulated in microaerobic conditions and decreased in aerobic conditions. Overexpression of *srrAB* resulted in nearly complete repression of TSST-1 production in both microaerobic and aerobic conditions. It is assumed that SrrA-SrrB acts in the global regulation of staphylococcal virulence factors, and may repress virulence factors under low-oxygen conditions. Furthermore, *srrAB* may provide a mechanistic link between respiratory metabolism, environmental signals, and regulation of virulence factors in *S. aureus* (Yarwood et al., 2001). This regulation is mediated in part by agr.

The Alternative Sigma Factor: Sigma B

In *S. aureus*, homologous genes to the *sigB* operon of the stationary-phase σ factor SigB (σB) of *Bacillus subtilis* were identified (Kullik and Giachino, 1997). The *sigB* region contains a total of six ORFs of which orf2, orf3, orf4 and orf5 show 64, 67, 71 and 77% similarity to the *B. subtilis* proteins RsbU, RsbV, RsbW and SigB, respectively, with SigB representing the σ factor and the Rsb proteins representing regulators of SigB. The organization of the *sigB* operon is shown in Fig. 19.

rsbU   rsbV   rsbW   sigB

Fig. 19. Organization and proposed function of the *sigB* operon in staphylococci. SigB is already present in unstressed cells, but it is sequestered by the anti-σ factor RsbW, which has RsbV kinase activity. RsbV is an anti-anti-σ factor, which can bind in its phosphorylated form to RsbW thus causing release of SigB from SigB-RsbW complex. RsbU has phosphatase activity. When RsbU gets activated, P-RsbV is dephosphorylated causing SigB release and interaction with the sigB specific promoters.

Very likely we have in *S. aureus* a similar sigB regulation cascade as described for *B. subtilis* (for review, see Mittenhuber, 2002). In *B. subtilis*, SigB is already present in unstressed cells (e.g., in exponential growth phase), but it is bound and sequestered by the anti-σ factor RsbW and therefore unable to interact with the core-enzyme of RNA polymerase (Hecker and Volker, 1998; Scott et al., 2000). A third protein, the anti-anti σ factor RsbV, which can also bind to RsbW, accomplishes release of SigB from RsbW. The phosphorylation status of RsbV is critical for its binding to RsbW. In its unmodified form, RsbV forms a complex with RsbW, whereas P-RsbV is unable to bind to RsbW that can then form a complex with SigB. This novel regulatory principle was called "partner switching" (Alper et al., 1994). Antagonistic activities of a kinase and two phosphatases control the phosphorylation status of RsbV. In addition, RsbW is also an RsbV kinase, whereas RsbU (phosphatase) dephosphorylates P-RsbV. Depending on the nature of the growth-restricting factor, RsbU is activated after imposition of environmental (physical) stress.

However, in *B. subtilis*, other genes are involved in stress regulation (e.g., *rsbS*, *rsbR*, *rsbT* and *rsbX*) that are not identified in *S. aureus* so far. Therefore, one has to be cautious in the unexamined functional adaptation of the homologous Rsb proteins.

Deletion of *sigB* in *S. aureus* revealed its function as a global regulator of virulence genes (Kullik et al., 1998a). The *sigB* mutants showed reduced pigmentation, accelerated sedimentation, and increased sensitivity to hydrogen peroxide during the stationary growth phase. Furthermore, the cytoplasmic alkaline shock protein 23 and pigmentation were undetectable, while some secreted enzymes such as thermonuclease and lipase were increased in the mutant. The *S. aureus rsbW* encodes an anti-σ factor of SigB and acts as a negative posttranslational regulator (Miyazaki et al., 1999). In an in vitro transcription runoff assay, RsbW prevented SigB-directed transcription from the sarA-P3 promoter, a known SigB-dependent promoter.

To find out the influence of a functional *sigB* on the global regulators sar and agr, studies were carried out in the *sigB*-positive MSSA1112 and Newman strains carrying the wildtype *rsbU* allele (Bischoff et al., 2001). The SigB concentration reached a maximum in the late exponential phase and declined towards the stationary phase when bacteria were grown in Luria-Bertani medium. In *sar*P1-3 reporter fusion studies, a strong SigB and growth phase-dependent increase in *sar* expression that was totally absent in either *rsbU* or *rsbU-sigB* deletion mutants was revealed. In contrast, expression of agr RNAIII is increased in the *sigB* mutant. Thus, SigB increases sar expression while simultaneously reducing the RNAIII level in a growth phase-dependent manner.

Interestingly, most *sigB* deletion phenotypes were only seen in *S. aureus* COL and Newman and not in 8325, which was found to contain an 11-bp deletion in the regulator gene *rsbU* and behaves phenotypically like a *sigB*-defective mutant. Since hydrogen peroxide (H_2O_2) represents an important stress factor for *S. aureus* during infection, it is postulated that *sigB* is important to survive high concentrations of peroxide especially during late stationary phase. The genes that are controlled by SigB (such as *asp23*) contain a SigB-specific promoter consensus sequence.

Critical Remarks

There is no doubt that the accessory gene regulator (agr) and the staphylococcal accessory regulator (sar) are central regulatory elements that control the production of virulence factors. Most of the functions of these global regulators have been defined using *S. aureus* RN6390, which is a representative of the laboratory strain 8325-4. However, RN6390 has a mutation in *rsbU* that results in a phenotype resembling that of a *sigB* mutant (Kullik et al., 1998b). For that reason, it remains unclear whether the regulatory characteristics described for RN6390 are representative for clinical isolates. To address this question, mutations were generated in the *sarA* and *agr* loci of three laboratory strains (RN6390, Newman, and S6C) and four clinical isolates (UAMS-1, UAMS-601, DB, and SC-1) and tested for collagen binding (*cna*-expression). The *sarA* mutants of strains UAMS-1 and UAMS-601 showed an increased capacity to bind collagen, while mutation of agr had little impact. Northern blot analysis confirmed that the increase in collagen binding was due to increased *cna* transcription. Without exception, an increased production

of proteases and a decreased capacity to bind fibronectin was revealed in *sarA* mutants. Mutation of *agr* had the opposite effect. Although *sarA* mutants had slightly reduced *fnbA* transcription, changes in the ability to bind fibronectin appeared to be more directly correlated with changes in protease activity. Lipase production was reduced in both *sarA* and *agr* mutants.

In contrast to the SigB-positive strains, mutation of *sarA* in RN6390 (affected in *rsbU*) resulted in a reduced hemolytic activity. The levels of the *sarC* transcript also seemed to be reduced in RN6390, but there was no difference in the overall pattern of *sarA* transcription or the production of SarA. Taken together, these results suggest that studies defining the regulatory roles of *sarA* and *agr* by using RN6390 are not always representative of the events that occur in clinical isolates of *S. aureus* (Blevins et al., 2002).

Ecology

Members of the genus *Staphylococcus* are widespread in nature and occupy a variety of niches. As a result of their ubiquity and adaptability, staphylococci are a major group of bacteria inhabiting the skin, skin glands, and mucous membranes of humans, other mammals, and birds. A variety of habitats present in the human cutaneous ecosystem can be distinguished by differences in the density and structure of the microbial communities inhabiting them, as well as by their anatomical and physiological properties. For example, skin regions supplied with large numbers of pilosebaceous units, sweat glands, and mucous membranes surrounding openings to the body surface contain the largest populations of staphylococci. In addition, staphylococcal communities may be found living in the follicular canals, the openings to sweat glands, the capacious lumen of sebaceous follicles, and on the surface of and beneath desquamating epithelial scales (Noble and Somerville, 1974; Noble and Pitcher, 1978).

Staphylococci may be found on the skin as residents or transients; as a result, one must use stringent criteria based on temporal studies and population size to estimate residency status and host range (Price, 1938; Noble and Somerville, 1974; Kloos and Musselwhite, 1975a). Resident bacteria are indigenous to the host, maintain relatively stable populations, and increase in numbers mainly by multiplication of those already present. Transient bacteria are derived from exogenous sources, found primarily on exposed skin, and may be easily washed away. Cross-contamination of staphylococci can occur readily where different host species come in contact with

one another, but where host specificity is high, the transient organisms will usually be eliminated within several hours or days, unless the normal defense barriers are compromised. Ideally, determination of natural host range should be made with host species that are relatively isolated in nature. Most of the ecological studies reported fall short of this ideal situation, but a few have indicated some clear patterns of host and niche preferences for certain *Staphylococcus* species.

Staphylococcus epidermidis (Schleifer and Kloos, 1975b) is the most prevalent and persistent *Staphylococcus* species on human skin. It is found over much of the body surface and produces the largest populations where moisture content and nutrition are high, such as in the anterior nares, axillae, inguinal and perineal area, and toe webs. This species may be found occasionally on other hosts, such as domestic animals, but it is presumably transferred there from human sources. *Staphylococcus hominis* (Kloos and Schleifer, 1975b) is also prevalent on human skin. Its population size is usually second or equal to *S. epidermidis* on skin sites where apocrine glands are numerous (e.g., in the axillae and inguinal and perineal areas). It can also colonize the drier regions of skin (e.g., on the extremities) more successfully than other species. *Staphylococcus haemolyticus* (Schleifer and Kloos, 1975c) shares many of the habitats of *S. hominis*, but it is usually found in smaller populations. Some individuals may carry unusually large populations of *S. haemolyticus*. A different subspecies of *S. haemolyticus* found living on nonhuman primate skin (e.g., *Pan*, *Macaca*, *Cercocebus*, *Erythrocebus*, *Microcebus* and *Lemur* skin) can be distinguished from the human-adapted subspecies on the basis of DNA-DNA hybridization (Kloos and Wolfshohl, 1979). Since it is difficult to distinguish colonies of the nonhuman primate *S. haemolyticus* subspecies from a sibling species, provisionally designated "*S. simians*," found also on nonhuman primate skin, adequate enumeration of this subspecies is not yet possible. *Staphylococcus warneri* (Kloos and Schleifer, 1975b) is found usually in small numbers on human skin, though a few individuals may carry unusually large populations. *Staphylococcus warneri* is a major species on nonhuman primates, especially on the more advanced Cercopithecoidea and Pongidae. Occasionally, small transient populations of *S. haemolyticus* or *S. warneri* may be isolated from domestic animals. *Staphylococcus capitis* (Kloos and Schleifer, 1975b) produces large populations on the human scalp following puberty. It is also found on other regions of the adult head, e.g., forehead, face, eyebrows, and external auditory

meatus in moderate-sized to large populations. The largest populations are found in areas where sebaceous glands are numerous and well developed. *Staphylococcus capitis* subsp. *ureolyticus* is present on regions of the head in rather small populations and, like *S. capitis*, may be found only occasionally on other body sites (Bannerman and Kloos, 1991). This subspecies has been isolated from both human and nonhuman primate skin (e.g., *Pan* skin).

   Staphylococcus caprae, originally isolated from the skin of domestic goats or in their milk (Devriese et al., 1983; Poutrel, 1984), has been isolated from human clinical specimens (Vandenesch et al., 1995; Kawamura et al., 1998). *Staphylococcus hominis* subsp. *novobiosepticus*, *S. lugdunensis*, *S. pasteuri*, *S. schleiferi* are other clinically significant species isolated from human specimens (Freney et al., 1988; Chesneau et al., 1993; Kloos et al., 1998b). Their original niche preference and prevalence is undetermined.

   Staphylococcus auricularis is one of the major species found living in the adult human, external auditory meatus and demonstrates a strong preference for this niche (Kloos and Schleifer, 1983a). A different subspecies of *S. auricularis* is found in the ear and specialized scent (or marking) glands of nonhuman primates (e.g., *Pan*, *Pongo*, *Cercopithecus*, *Lemur*, *Galago* and *Microcebus*; Kloos, 1985; Kloos and Schleifer, 1986).

   Staphylococcus aureus is a major species of primates, though specific ecovars or biotypes can be found occasionally living on different domestic animals or birds (Meyer, 1967; Kloos, 1980). This species is found infrequently on nonprimate wild animals. In humans, *S. aureus* has a niche preference for the anterior nares, especially in the adult. Here it can exist as a resident or as a transient member of the normal flora. *Staphylococcus aureus* selectively adheres to nasal epithelial (mucosal) cells (Aly et al., 1981). Nasal carrier rates range from less than 10% to more than 40% in normal adult human populations residing outside of the hospital (Noble and Somerville, 1974). The nasal adherence of *S. aureus* is significantly greater for carriers of this species than for noncarriers. *Staphylococcus aureus* subsp. *anaerobius* is found living on sheep (De la Fuente et al., 1985).

   The host range of *Staphylococcus saprophyticus* and similar species varies from humans to lower mammals and birds (Kloos, 1980; Devriese, 1986). As a group, these staphylococci are most prevalent on lower primates and mammals. Those species found most frequently on primates include *S. saprophyticus*, *S. cohnii* and *S. xylosus*. *Staphylococcus saprophyticus* is found usually in small, transient populations on the skin of humans or other primates. This species possesses surface properties that allow it to adhere readily to urogenital cells (Colleen et al., 1979). It may also be isolated from lower mammals and environmental sources. *Staphylococcus saprophyticus* subsp. *bovis* is found in the anterior nares of cows (Hájek et al., 1996). *Staphylococcus cohnii* (Schleifer and Kloos, 1975c) is found as a temporary resident or transient on human skin, and *S. cohnii* subsp. *urealyticus* (Kloos and Wolfshohl, 1991) is sometimes found on human skin, but it is often one of the major species and subspecies of nonhuman primates, especially the lower primates. The largest populations of subspecies are found living on the Tupaiidae, Prosimii, and Ceboidea (Kloos and Wolfshohl, 1983b). A third subspecies is also found on the Ceboidea. *Staphylococcus xylosus* (Schleifer and Kloos, 1975c) is often found as a transient on the skin of lower primates and other mammals, and occasionally on birds (Kloos et al., 1976b; Akatov et al., 1985; Devriese et al., 1985). The related species *S. kloosii* has been found living on a variety of lower mammals including wild marsupials, rodents and carnivores, and less frequently on domestic animals (Schleifer et al., 1984; Kloos, 1980). *Staphylococcus arlettae* has been isolated from poultry and goats, *S. equorum* from horses, and *S. gallinarum* from poultry (Devriese et al., 1983; Schleifer et al., 1984).

   Staphylococcus intermedius is a major species of the domestic dog (Hájek and Marsalek, 1976b; Krogh and Kristensen, 1976). This species can be found in relatively large populations on canine skin and can on occasion be transferred to the skin of human handlers (Kloos et al., 1976b). *Staphylococcus intermedius* appears to be also indigenous to a variety of other carnivores, including the mink (*Mustela*; Hájek et al., 1972; Oeding et al., 1973; Hájek, 1976a), fox (*Vulpes*; Hájek, 1976a), and raccoon (*Procyon*; Kloos et al., 1976b). It has also been isolated from horses and pigeons (Hájek and Marsalek, 1971; Hájek, 1976a). *Staphylococcus felis* is one of the major species of the domestic cat (Igimi et al., 1989). *Staphylococcus schleiferi* subsp. *coagulans* is a coagulase-positive species, which has been isolated from the external auditory meatus of dogs with ear infections (Igimi et al., 1990). Other coagulase-positive species isolated from animals include *S. delphini* (Varaldo et al., 1988) and *S. lutrae* (Foster et al., 1997). *Staphylococcus sciuri*, *S. sciuri* subsp. *carnaticus* and *S. sciuri* subsp. *rodentium* have been isolated from a variety of lower mammals and domestic animals (Kloos et al., 1976a; Kloos et al., 1997). In addition, the *S. sciuri* subspecies may be isolated from human clinical specimens (Marsou et al.,

1999). *Staphylococcus scuiri* appears to be a natural reservoir of methicillin resistance and staphylolytic enzyme genes (Kloos et al., 1997). Their role, if any, in the appearance of methicillin-resistance in the more pathogenic species *S. aureus* has not yet been determined. *Staphylococcus hyicus* and *S. chromogenes* are found predominantly on domestic ungulates such as pigs, cattle and horses (Devriese et al., 1978; Devriese, 1979a; Devriese, 1986; Phillips et al., 1980; Hájek et al., 1986). *Staphylococcus lentus* (Schleifer et al., 1983) has been isolated in large populations from domestic sheep and goats (Kloos et al., 1976a; Kloos et al., 1976b), occasionally from other domestic animals (Devriese et al., 1985), and is a major bacterium in saliva of rabbits (Kanda et al., 2001). Members of the newly described genus *Macrococcus* are found primarily on domestic animals (Kloos et al., 1998a).

Staphylococci have been isolated sporadically from a wide variety of environmental sources such as soil, beach sand, seawater, fresh water, plant surfaces and products, feeds, meat and poultry, dairy products, and on the surfaces of cooking ware, utensils, furniture, clothing, blankets, carpets, linens, paper currency, and dust and air in various inhabited areas. *Staphylococcus carnosus*, *S. carnosus* subsp. *utilis*, *S. condimenti*, *S. fleurettii*, *S. piscifermentans* and *S. vitulus* have been isolated from various food products (Schleifer and Fischer, 1982; Tanasupawat et al., 1992; Webster et al., 1994; Probst et al., 1998; Vernozy-Rozand et al., 2000). Some of the staphylococci may be involved in food poisoning and spoilage or in desirable food fermentation. Recently, a staphylococcal species from Dominican amber, *S. succinus*, has been described (Lambert et al., 1998).

With the exception of the animal products, most environmental sources contain small, transient populations of staphylococci, many of which are probably contaminants disseminated by human, animal, or bird host carriers. Possibly, certain species (e.g., *S. sciuri* and *S. xylosus*) can grow in habitats containing only an inorganic nitrogen source, and thus might be more free-living than other staphylococci (Emmett and Kloos, 1975; Emmett and Kloos, 1979). These species have been isolated in small numbers from beach sand, natural waters, and marsh grass (Kloos and Schleifer, 1981) and also from plant products (Bucher et al., 1980; Pioch et al., 1988). Flies of the genera *Musca*, *Fannia* and *Stomoxys*, commonly found in human and animal habitations, can carry populations of staphylococci (e.g., *S. muscae*; Hájek et al., 1992) and appear to be significant vectors of these organisms in an epizootiological chain (Hájek and Balusek, 1985).

Opportunistic Pathogens

The coagulase-positive species *S. aureus*, *S. intermedius*, *S. delphini*, *S. schleiferi* subsp. *coagulans* and the coagulase-variable species *S. hyicus* are regarded as potentially serious pathogens. *Staphylococcus aureus*, since its early discovery as an opportunistic pathogen, continues to be a major cause of mortality and is responsible for a variety of infections. In the late 1950s and early 1960s, *S. aureus* caused considerable morbidity and mortality as a nosocomial pathogen. Among the major human infections caused by this species are furuncles, carbuncles, impetigo, toxic epidermal necrolysis (scalded skin syndrome), pneumonia, osteomyelitis, acute endocarditis, myocarditis, pericarditis, enterocolitis, mastitis, cystitis, prostatitis, cervicitis, cerebritis, meningitis, bacteremia, toxic shock syndrome, and abscesses of the muscle, skin, urogenital tract, central nervous system, and various intraabdominal organs. In addition, staphylococcal enterotoxin is involved in food poisoning. Methicillin-resistant *S. aureus* (MRSA) strains have emerged in the 1980s as a major clinical and epidemiological problem in hospitals. These strains are beginning to spread out of the hospitals and into communities. The origin and future significance of these isolates to the community has yet to be determined.

Staphylococcus aureus can also infect a variety of other mammals and birds. The more common natural infections include mastitis, synovitis, arthritis, endometritis, furuncles, suppurative dermatitis, pyemia, and septicemia. Staphylococcal mastitis in either a clinical or subclinical form may have considerable economic consequences in the dairy industry. *Staphylococcus aureus* subsp. *anaerobius* is the etiologic agent of an abscess disease in sheep, symptomatically similar to caseous lymphadenitis (De la Fuente et al., 1985). Hagan and Bruner's *Microbiology and Infectious Diseases of Domestic Animals* (Timoney et al., 1988) may be referenced for further information on the nature of *S. aureus* infections in animals.

Staphylococcus intermedius is a serious opportunistic pathogen of dogs and may cause otitis externa, pyoderma, abscesses, reproductive tract infections, mastitis, and purulent wound infections. *Staphylococcus hyicus* has been implicated as the etiologic agent of infectious exudative epidermitis (greasy pig disease) and septic polyarthritis of pigs, skin lesions in cattle and horses, osteomyelitis in poultry and cattle, and occasionally associated with mastitis in cattle. *Staphylococcus delphini* has been implicated in purulent skin lesions of dolphins (Varaldo et al., 1988). *Staphylococcus schleiferi* subsp. *coagulans* is

associated with external auditory meatus of dogs (Igimi et al., 1990).

Although the coagulase-negative staphylococcal species constitute a major component of the normal microflora in humans, their role (especially that of *S. epidermidis*) in causing nosocomial infections has been recognized and well documented over the last two decades. The increase in infections by these organisms has been correlated with the wide medical use of prosthetic and indwelling devices and the growing number of immunocompromised patients in hospitals. Infectious processes may result from the introduction of endogenous staphylococci beyond the normal integumentary barriers. *Staphylococcus epidermidis* appears to have the greatest pathogenic potential and adaptive diversity. This species has been implicated in bacteremia, native and prosthetic valve endocarditis, osteomyelitis, pyoarthritis, peritonitis during continuous ambulatory dialysis, mediastinitis, infections of permanent pacemakers, vascular grafts, cerebrospinal fluid shunts, prosthetic joints, and a variety of orthopedic devices, and urinary tract infections including cystitis, urethritis, and pyelonephritis. Recent reviews have been published on the nature of human infections caused by *S. epidermidis* and other coagulase-negative species (Kloos and Bannerman, 1994; Rupp and Archer, 1994; Crossley and Archer, 1997). Nosocomial methicillin-resistant *S. epidermidis* (MRSE) strains became a serious clinical problem in the 1980s, especially in patients with prosthetic heart valves or who had undergone other forms of cardiac surgery (Archer and Tenenbaum, 1980; Karchmer et al., 1983). *Staphylococcus epidermidis* has also been occasionally associated with mastitis in cattle (Baba et al., 1980; Devriese and De Keyser, 1980; Holmberg, 1986).

Certain other coagulase-negative species have been associated with infections in humans and animals. *Staphylococcus haemolyticus* is the second most frequently encountered species of this group found in human clinical infections. It has been implicated in native valve endocarditis, septicemia, peritonitis, and urinary tract infections, and is occasionally associated with wound, bone, and joint infections. *Staphylococcus haemolyticus* has been occasionally associated with mastitis in cattle (Baba et al., 1980). *Staphylococcus caprae*, previously misidentified as *S. haemolyticus*, *S. hominis* and *S. warneri*, is widely distributed in human clinical specimens (Kawamura et al., 1998) and has been implicated in cases of infective endocarditis, bacteremia, and urinary tract infections. *Staphylococcus lugdunensis* has been implicated in native and prosthetic valve endocarditis, septicemia, brain abscess, and chronic osteoarthritis

and infections of soft tissues, bone, peritoneal fluid, and catheters, especially in patients with underlying diseases. *Staphylococcus schleiferi* has been implicated in human brain empyema, osteoarithritis, bacteremia, wound infections, and infections associated with a cranial drain and jugular catheter. This species occurs less frequently than *S. lugdunensis* in the hospital environment and human infections. *Staphylococcus saprophyticus* is an important opportunistic pathogen in human urinary tract infections, especially in young, sexually active females. It is considered to be the second most common cause of urinary tract infections, such as acute cystitis or pyelonephritis, in these patients. This species can also produce in men urinary tract infections, which (unlike those in women) occur most commonly in the elderly with predisposing diseases of the urinary tract (Marrie et al., 1982a; Hovelius et al., 1984; Hovelius, 1986). *Staphylococcus saprophyticus* has occasionally been isolated from wound infections and septicemia (Marsik and Brake, 1982; Fleurette et al., 1987).

Several other coagulase-negative species have been implicated at low incidence in a variety of human infections. In most cases, patients with these infections had predisposing or underlying diseases that drastically altered their immune systems, and had also experienced surgery or intravascular manipulations. *Staphylococcus warneri* has been on occasion the etiologic agent of vertebral osteomyelitis, native valve endocarditis, and urinary tract infections in males and females. This species has been associated with mastitis in cattle (Devriese and Derycke, 1979b; Devriese and De Keyser, 1980). *Staphylococcus simulans* has been associated with human chronic osteomyelitis, pyarthrosis, and bovine mastitis. *Staphylococcus felis*, a relative of *S. simulans*, has been isolated from clinical infections in cats, including external ear otitis, cystitis, abscesses, wounds, and other skin infections (Igimi et al., 1989). *Staphylococcus capitis* has been implicated in endocarditis, septicemia, and catheter infections. *Staphylococcus hominis* has been associated with human endocarditis, peritonitis, septicemia, and arthritis. Some of the earlier reports indicating an association of this species with infections were in error, owing to the misidentification of phosphatase-negative strains of *S. epidermidis* as *S. hominis*. *Staphylococcus cohnii* has been associated with urinary tract infections and arthritis. *Staphylococcus chromogenes*, a close relative of *S. hyicus*, is commonly isolated from the milk of cows suffering from mastitis, although its role as an etiologic agent is questionable (Devriese and De Keyser, 1980; Langlois et al., 1983; Watts et al., 1984). *Staphylococcus sciuri* subspecies has been iso-

lated from wound, skin, and soft tissue infections (Marsou et al., 1999).

Members of the *Macrococcus* genus constitute a portion of the normal microflora of cattle, horses and ponies (Kloos et al., 1998a). *Macrococcus caseolyticus* has been isolated from abscesses of slaughtered lambs (de la Fuente et al., 1992).

Applications

It has been known that Gram-positive and catalase-positive cocci play an important role in the ripening process of dry sausages (Lerche and Sinell, 1955; Niinivaara and Pohja, 1956). The predominant microorganism in fermented meat is *S. carnosus*, appearing in the early literature as *Micrococcus* (Schleifer and Fischer, 1982). For more than 50 years, *S. carnosus* has been used alone or in combination with lactobacilli or pediococci, as a starter culture for the production of raw fermented sausages. One of the main advantages of starter cultures in fermented food processing is that the fermentation and ripening process can be carried out under controlled conditions. In this way, food-poisoning and food-spoilage microorganisms can be suppressed, and the course of the fermentation process and its termination more reliably monitored. During the ripening process of dry sausage, *S. carnosus* exerts several desired functions (Liepe and Porobic, 1983; Götz, 1990). First, *S. carnosus* gradually reduces nitrate to nitrite and nitrite to ammonia. The advantage of this reaction is that the nitrate concentration is lowered, and that nitrite can combine with myoglobin to form nitrosomyoglobin, which gives the typical red color. As outlined above, nitrite is further reduced to the ammonia thus lowering the unbound nitrite concentration (Neubauer and Götz, 1996). Other advantages are development of characteristic flavor, lowering the pH moderately, and the capacity to reduce hydrogen peroxide produced by the catalase-negative lactobacilli, thus preventing odors.

Another application of *S. carnosus* is as an alternative host organism for the production of heterologous proteins or hormones. Transformation systems (Götz and Schumacher, 1987; Augustin and Götz, 1990) have been worked out and useful plasmid vectors have been constructed (Wieland et al., 1995; Peschel et al., 1996). With the aid of the lipase propeptide, an efficient secretion system has been worked out (Götz and Rosenstein, 2001b), which has been used successfully for secretion of heterologous proteins such as the human growth hormone (Sturmfels et al., 2001).

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CHAPTER 1.2.2

The Genus *Streptococcus*—Oral

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The classification of the genus *Streptococcus* has undergone major revisions since the 1980s as a result of the application of molecular and chemotaxonomic approaches (Schleifer and Kilpper-Bälz, 1987). As a result, two new genera, *Enterococcus* and *Lactococcus*, were created from species previously allocated to the enterococcus (or "fecal") and the lactic groups of streptococci, respectively (Sherman, 1937; Jones, 1978; Hardie, 1986a). In addition, some other species previously considered to be streptococci, such as the anaerobes *S. hansenii*, *S. morbillorum*, *S. parvulus* and *S. pleomorphus*, have been excluded from the genus (Schleifer and Kilpper-Bälz, 1987; Hardie and Whiley, 1997).

The species that remain within the genus *Streptococcus* following these taxonomic revisions include those that Sherman (1937) described as the viridans group, and for which others favor the term "oral streptococci" (Jones, 1978; Hardie, 1986b). The type of hemolysis produced by these streptococci when grown on blood agar varies considerably, both between and within species, so that strains may display either complete (β), partial (α), or no (γ) hemolysis. In view of this variation, the term "viridans" would seem to be rather misleading for the group as a whole, although it is still commonly used. Since these streptococci are found predominantly, but not exclusively, in the mouth and upper respiratory tract, the continued use of the term "oral streptococci," as suggested in *Bergey's Manual of Systematic Bacteriology* (Hardie, 1986a; Hardie, 1986b) is perhaps justified and convenient for descriptive purposes, although it has no particular taxonomic validity.

In addition to comprising part of the normal body flora, the oral streptococci are also involved in a number of human diseases, usually as opportunistic pathogens. As described below, members of the mutans group are particularly associated with dental caries, members of the anginosus group occur frequently in purulent infections in various parts of the body, and almost all species have been reported as etiological agents in infective endocarditis. In addition, it is now recognized that several species can on occasion be significant pathogens in immunologically compromised patients.

Identification of the oral streptococci to species level has been problematic in the past, partly because of taxonomic and nomenclatural uncertainties. However, in the light of current knowledge, it should be possible to identify most isolates with a reasonable degree of confidence. Only by proper characterization of these organisms will it be possible to elucidate fully their ecological distribution, transmission, and role in infectious processes.

In this chapter consideration is given to the habitat, distribution, isolation, and taxonomy of the oral streptococci, and to their role in human disease. Although *S. pneumoniae* should be regarded as one of the oral streptococci on the basis of molecular and numerical taxonomic studies, rather than belonging to the pyogenic group (Schleifer and Kilpper-Bälz, 1987), this species is not considered in detail here. Medical aspects of the genus are discussed in the chapter *Streptococcus pneumoniae* in this Volume.

In regard to the nomenclature of streptococci, several species epithets were changed (Trüper and De Clari, 1997; Trüper and De Clari, 1998) in a recent attempt to adhere strictly to the correct naming of species according to Rule 12c of the International Code of Nomenclature of Bacteria (Lapage et al., 1992). These included some names long-established within the oral streptococci, with *Streptococcus cricetus*, *S. crista*, *S. rattus*, *S. sanguis* and *S. parasanguis* changed to *S. criceti*, *S. cristatus*, *S. ratti*, *S. sanguinis* and *S. parasanguinis*, respectively. This development has been argued against and the risk of confusion from changing longstanding names highlighted (Kilian, 2001). The Judicial Commission of the International Committee on Systematic Bacteriology recently ruled (Amendment to Rule 61 of the Bacteriological Code, Minute 7, Session 1 of the meeting of the Judicial Commission held 14, 15 and 18th August 1999, Sydney, Australia; Judicial Commission of the International Committee on Systemic Bacteriology, 2000) that priority be given to the stabilization of nomenclature over orthographic correctness and that names on the

Approved Lists of Bacterial Names, the Validation Lists and the Notification Lists should not be changed on grammatical grounds. Consequently in this chapter we have used the names of species as originally validly published and have not adopted the name changes proposed by Trüper and De Clari (Trüper and De Clari, 1997; Trüper and De Clari, 1998).

Habitat and Distribution

The oral or viridans streptococci form an important component of the normal microbial flora of the mouth and the upper respiratory tract of humans (Hardie and Marsh, 1978b; Jones, 1978). These sites appear to be their main habitats, although some species can be isolated from other body sites and from feces (Van Houte et al., 1971; Unsworth, 1980) and have also been found in soil (Gledhill and Casida, 1969). Several of the species found in humans have also been isolated from the dental plaque of animals (Dent et al., 1978).

Within the oral cavity, a variety of surfaces and ecological niches are available for colonization, and it is well established that different species of streptococci (and other bacteria) preferentially become established at different sites (Hardie and Bowden, 1974b; Marsh and Martin, 1999). Thus, for example, *S. salivarius* is found in relatively high numbers on the dorsal surface of the tongue and in saliva but not in dental plaque, whereas *S. sanguis* preferentially colonizes the tooth surface, and *S. vestibularis* appears to favor the vestibular mucosa.

The naturally occurring proportions of different species on particular types of surface in the mouth, including the nonshedding, hard surfaces of the teeth and the various types of mucous membrane, have been shown to correlate with their experimentally observed adherence to such surfaces (Gibbons and Van Houte, 1975; Gibbons, 1984). The mechanisms by which these bacteria adhere to surfaces have been studied extensively (Ellwood et al., 1979; Beachey, 1980; Berkeley et al., 1980; Ten Cate et al., 1984). It is thought that bacteria have specific surface ligands or adhesins on their surfaces, which enable them to bind to complementary host tissue components. The adhesins often possess lectin-like or hydrophobic properties and may be present on filamentous surface appendages like pili or fimbriae (Gibbons, 1984; Weerkamp et al., 1984; Mergenhagen et al., 1987). The relationship of surface structures to adhesion, coaggregation with other bacteria, and hydrophobicity have been studied in detail in some species, including *S. salivarius* (Weerkamp and Jacobs, 1982; Handley et al., 1984; Handley et al., 1987) and *S.*

sanguis (Handley et al., 1985), and the ultrastructural characteristics of the complex of different surface appendages defined. Study of the growth of *S. salivarius* strain HB and of four adhesion-deficient mutants under different conditions in a chemostat indicated that the ability to adhere to buccal epithelial cells and to coaggregate with *Veillonella parvula* did not vary with growth rate, whereas cell surface thickness, hydrophobicity, and cell surface proteins of these strains were phenotypically variable characteristics (Harty and Handley, 1989).

Both sucrose-independent and sucrose-dependent mechanisms are involved in the adherence of *S. mutans* to tooth surfaces (Gibbons, 1984; Koga et al., 1986). Extracellular glucan synthesis is not essential for initial attachment, as once believed, but is important in subsequent retention and accumulation of plaque deposits. As described below, mutant strains lacking the enzymes responsible for production of these polymers have reduced caries-inducing potential in experimental animals (Koga et al., 1986; Loesche, 1986).

A group of cell-surface-anchored polypeptides in oral streptococci, referred to as the "antigen I/II family," are considered to be particularly significant in adhesion to oral surfaces (Jenkinson and Demuth, 1997). Cell surface proteins SspA and SspB in *S. gordonii* and SpaP in *S. mutans* are examples of the I/II family of peptides, and have been shown to bind to collagen. These may be critical for the intratubular growth of streptococci in human root dentinal tubules, and thus, have significance in the establishment of endodontic (root canal) infections (Love et al., 1997).

An update on many aspects of oral biofilm formation and the properties of dental plaque can be found in a recently published book (Newman and Wilson, 1999). Studies on genes that code for biofilm phenotypes (Loo et al., 2000) and amylase-binding proteins (Rogers et al., 1998) in *S. gordonii* are examples of the increasing application of molecular methods in this field.

Isolation and Cultivation

Nutritional Requirements

The nutritional requirements of oral streptococci, as with all facultative anaerobic streptococci, include amino acids, peptides and proteins, a carbohydrate source, fatty acids, vitamins, and purines and pyrimidines, in addition to inorganic ions. These requirements necessitate the use of complex media that often contain meat extract. In addition, an elevated CO_2 level (typically 5%) during incubation is essential for the growth of

several species, including *S. mutans*, strains of the anginosus group, and *S. pneumoniae*.

Sampling of Oral Streptococci

Oral streptococci may be isolated from almost any type of clinical specimen, including blood cultures, pus, wound or surface swabs, body fluids, and biopsies. When taking material from the mouth and upper respiratory tract, soft tissue surfaces can be sampled with a cotton swab and whole saliva (its flow possibly stimulated by chewing a piece of sterile paraffin wax) can be dribbled into a sterile bottle or tube. As an alternative to collecting saliva, particularly in infants, quantitatively similar results can be obtained by taking a sample from the dorsal surface of the tongue with a standard disposable plastic loop (Beighton, 1986).

For collection of samples of dental plaque, it is usually necessary to use a rigid instrument of some kind to scrape the adherent material from the tooth surface, although dental floss can be used for interproximal areas between the teeth. A variety of techniques and instruments have been used by different investigators, including dental probes, scalers, curettes, abrasive strips, hypodermic needles, wires, wood sticks, and paper points (Hardie and Bowden, 1976c).

Transport Medium for Oral Streptococci

If immediate laboratory processing of a sample is not possible, a suitable transport medium is required. The reduced transport fluid (RTF) of Syed and Loesche (1972) is often used and is suitable for holding a clinical sample or streptococcal population at room temperature.

Reduced Transport Fluid (RTF)

Na_2CO_3 (8% solution; filter-sterilized)	0.5 ml
0.1 M Ethylenediamine tetraacetate (EDTA),	1.0 ml
DL-Dithiothreitol (Cleland's reagent; filter-sterilized)	2.0 ml
Solution a: 0.6% K_2HPO_4	7.5 ml
Solution b: 0.6% KH_2PO_4, 1.2% NaCl, 1.2% $(NH_4)_2SO_4$, and 0.24% $MgSO_4$	7.5 ml

Dilute up to 100 ml with deionized water. Filter sterilize.

Routine Cultivation of Oral Streptococci

Oral streptococci can be maintained in the laboratory by culture on a variety of blood-containing agar media, such as oxoid blood agar no. 2 (Oxoid Ltd., Hants, United Kingdom) with 5% horse blood. For liquid culture, commercially available Todd-Hewitt broth (Oxoid Ltd., Hants, United Kingdom), brain-heart infusion broth (Difco Laboratories, Michigan, United States), and other formulations may be used. The following broth medium (strep. base plus 0.5% glu-

cose) has been used for many years in the authors' laboratory (Hardie and Bowden, 1974a; Whiley and Hardie, 1989):

Strep Base

Proteose peptone (Oxoid)	20 g
Yeast extract (Difco)	5 g
NaCl	5 g
Na_2HPO_4	1 g
Glucose	5 g

Dissolve ingredients in distilled water and dilute up to 1 liter; adjust pH to 7.6. Autoclave at 121°C for 15 min

Isolation of Extracellular Polysaccharide-Producing Streptococci

Two of the most commonly used selective isolation media for oral streptococci contain sucrose, and these allow some species to produce characteristic colonies as a result of extracellular polysaccharide formation from this substrate. The recipes for these agar media (TYC and MS) are given below:

Trypticase-Yeast Extract-Cystine (TYC) 5% Sucrose Agar (De Stoppelaar et al., 1967)

Trypticase (BBL)	15.0 g
Yeast extract (Difco)	5.0 g
L-Cystine	0.2 g
Na_2SO_3	0.1 g
NaCl	1.0 g
$NaHCO_3$	0.1 g
NaCl	1.0 g
$Na_2HPO_4 \cdot 12H_2O$	2.0 g
$NaHCO_3$	2.0 g
Sodium acetate ($\cdot 3H_2O$)	20.0 g
Sucrose	50.0 g
Agar	12.0 g

Dissolve in 1 liter of deionized water. Adjust pH to 7.3. Autoclave at 121°C for 15 min.

Commercial TYC is marketed by Lab M, IDG (UK) Limited, Bury, Lancashire, United Kingdom), but substituting trypticase (BBL) and yeast extract (Difco) with Lab M tryptone and yeast extract, respectively

COLONIAL MORPHOLOGY OF ORAL STREPTOCOCCI ON TYC AGAR Several species of oral streptococci give rise to characteristic colonial morphologies on TYC agar that may be useful as an aid to identification (Hardie and Marsh, 1978a). However, it must be emphasized that the presumptive identification of a strain should always be backed up by additional biochemical criteria.

Descriptions of the colonial morphology of some oral streptococci on TYC agar are given below:

Streptococcus mutans form rough, heaped, irregular colonies, resembling frosted glass. Although mostly crumbly, whole colonies can be picked off the agar. White, gray or yellow in color, colonies are 0.5–2.0 mm in diameter and

may have a drop of liquid (water-soluble glucan) on top or a puddle of polysaccharide around the colony.

Streptococcus sobrinus form rough, irregular colonies, less heaped than those of *S. mutans*. Colonies are white, 0.5–2.0 mm in diameter, and surrounded by a white halo or milky zone in the agar.

Streptococcus sanguis form smooth or rough, hard and rubbery colonies (gray, white, or colorless, 1–3 mm in diameter) that adhere strongly to the agar making them difficult to remove with a loop. Some strains do not produce extracellular polysaccharide.

Streptococcus oralis produce two colony types: 1) a hard type similar to *S. sanguis* and 2) a soft, smooth, nonadherent type. Colonies are gray, white, or colorless, and 0.5–2.0 mm in diameter. Polysaccharide production is a variable characteristic of this species.

Streptococcus anginosus and related taxa form rough, dry, crumbly, or smooth and soft colonies (white or gray, 1–2 mm in diameter). No polysaccharide is produced.

Streptococcus vestibularis form smooth, soft, white colonies, 1–2 mm in diameter. No extracellular polysaccharide is produced.

Streptococcus gordonii colonies of polysaccharide-producing strains resemble those of *S. sanguis*. Nonpolysaccharide-producing strains are also encountered.

Streptococcus mitis and *S. parasanguis* consist of nonpolysaccharide-producing strains. Colonial morphology is similar to soft *S. oralis* strains, with no distinctive features.

Streptococcus salivarius colonies (white or gray, 2–6 mm in diameter) are domed, smooth and mucoid. Older colonies may become hard, pitting the agar.

Isolation and Colony Morphology on Mitis Salivarius (MS) Agar   This widely used agar is for the selection of oral streptococci and contains 5% sucrose, trypan blue, crystal violet, and potassium tellurite as selective agents. Commercial sources include BBL and Difco. One recipe (Difco) is detailed below:

Mitis Salivarius (MS) Agar

Bacto tryptose (Difco)	10 g
Proteose peptone no. 3 (Difco)	5 g
Proteose peptone (Difco)	5 g
Bacto dextrose (Difco)	1 g
Bacto saccharose (Difco)	50 g
K_2HPO_4	4 g
Trypan blue	0.075 g
Bacto crystal violet (Difco)	0.0008 g
Bacto agar (Difco)	15 g

Dissolve by heating in deionized water to boiling and dilute up to a total of 1 liter. Sterilize at 121°C for 15 min. Cool to 50–55°C. Add 1 ml of 3.5% potassium tellurite.

When grown on MS agar, strains of *S. mutans* produce rough colonies that often look like frosted glass in appearance. *Streptococcus salivarius* produces either large (2–5 mm diameter), mucoid, smooth colonies (resembling gum drops) or rough, irregular colonies; *S. sanguis* can form hard, rubbery, adherent colonies (called "zooglea") of less than 2 mm in diameter. *Streptococcus vestibularis* strains grown anaerobically on MS agar produce matte colonies (2–3 mm in diameter) with undulate edges

Selective Media for *S. mutans*

Most media developed for selection of mutans streptococci have been based on either MS agar or TYC agar, but contain bacitracin and increased amounts of sucrose.

Two frequently used examples (one MS agar-based and the other TYC-based) are:

Mitis salivarius sucrose bacitracin (MSB; Gold et al., 1973): MS agar plus 15% sucrose plus bacitracin (0.2 units/ml).

Trypticase, yeast extract, cystine (TYC) agar plus 15% (w/v) sucrose plus 0.1 unit/ml bacitracin (TYCSB; Van Palenstein Helderman et al., 1983). A variation has been described (Wade et al., 1986) on which optimum recovery/growth of both *S. mutans* (strain National Collection of Type Cultures [NCTC] 10449) and *S. sobrinus* (strain 6715) was achieved. This medium consisted of TYC agar plus 15% (w/v) additional sucrose and 0.2 unit of bacitracin per ml (compared to 0.1 unit per ml in the original TYCSB formulation).

Although TYCSB was developed in response to the shortcomings of MS-based media (Van Palenstein Helderman et al., 1983), the increased recovery of *S. mutans* initially reported has not always been verified in other studies (Beighton et al., 1989).

Other selective media for *S. mutans* that have been described include: MS agar plus a final concentration of 40% sucrose (MS40S; Ikeda and Sandham, 1972); mannitol-sorbitol-fuchsin-azide agar (MSFA; Linke, 1977); and glucose-sucrose-tellurite-bacitracin agar (GSTB; Tanzer et al., 1983).

Semiselective Medium for the Anginosus Group Species

NAS medium (Whiley et al., 1993) is nalidixic acid sulfamethazine-containing agar (sensitivity agar, STA Lab 12, Lab M Amersham, Bury, United Kingdom).

STA Lab 12

Peptone-infusion solids	21.5 g
Starch	0.6 g
Sodium chloride	5.0 g
Disodium citrate	1.0 g
Adenine sulfate	0.01 g
Guanine hydrochloride	0.01 g
Uracil	0.01 g
Xanthine	0.01 g
Aneurine (thiamin) hydrochloride	0.01 g
Agar no. 2	12.0 g

Dissolve in and dilute up to 1 liter with deionized water. Sterilize at 121°C for 15 min. Cool to 50°C. Add nalidixic acid (30 µg/ml; Sigma Chemical Co. Ltd. Poole, Dorset, United Kingdom), sulfamethazine (4-amino-*N*-[4,6-dimethyl-2-pyrimidyl]-benzene-sulfonamide; 1,000 µg/ml; Sigma), and 5% (v/v) defibrinated horse blood.

Colonies are approximately 1 mm in diameter, gray, entire and convex. NAS medium will also support the growth of *S. mutans* and *S. sobrinus*. *Streptococcus mutans* colonies are easily differentiated from anginosus group species by the production of flat, 1 mm in diameter, dry, pitting colonies.

Phylogeny of Oral Streptococci

16S rRNA gene sequence analyses (Bentley et al., 1991; Kawamura et al., 1995a; Kawamura et al., 1995b) have shown that the oral streptococci are included within four multispecies phylogenetic units: the anginosus, mitis, mutans and salivarius groups. Together with the pyogenic group, bovis group and several unaffiliated species these constitute the genus *Streptococcus* sensu stricto and form part of the low G+C (*Clostridium-Bacillus*) branch of the Gram-positive eubacteria (Ludwig et al., 1985; Schleifer and Ludwig, 1995). Currently, member species of these phylogenetic units include: *S. anginosus*, *S. constellatus*, *S. intermedius* (anginosus group); *S. mitis*, *S. oralis*, *S. pneumoniae*, *S. sanguis*, *S. parasanguis*, *S. gordonii*, *S. crista*, *S. peroris*, *S. infantis*, and *S. australis* (mitis group); *S. mutans*, *S. sobrinus*, *S. rattus*, *S. cricetus*, *S. downei*, *S. macacae, S. orisratti* and *S. ferus* (mutans group); *S. salivarius*, *S. vestibularis*, *S. thermophilus* (salivarius group; Table 1).

Phylogenetic inference based on an alternative data source (internal fragment of the manganese-dependent superoxide dismutase gene $sodA_{INT}$; Poyart et al., 1998) is in broad agreement with conclusions based on the 16S rDNA sequence data, with the formation of the same phylogenetic units, the species within the mutans group forming relatively distinct lines of descent and the anginosus group seen to be closely related to the mitis group.

Table 1. Species groups within the oral streptococci.

Group	Species	G+C content (mol%)[a] of DNA
Mutans	*S. mutans*	36–38
	S. sobrinus	44–46
	S. cricetus	42–44
	S. rattus	41–43
	S. macacae	35–36
	S. downei	41–42
	S. ferus	43–45
	S. orisratti	39–43
Salivarius	*S. salivarius*	37–40
	S. vestibularis	38–41
	S. thermophilus	37–40
Anginosus	*S. anginosus*	38–40
	S. intermedius	38–41
	S. constellatus	
	subsp. *constellatus*	37–38
	subsp. *pharyngis*	35
Mitis	*S. mitis*	40–41
	S. oralis	38–42
	S. gordonii	38–42
	S. infantis	40
	S. peroris	40
	S. pneumoniae	36–37
	S. crista	43
	S. parasanguis	41–43
	S. australis	43
	"tufted mitior"	42–43

[a]G+C content values are to the nearest percent.

Taxonomy of Oral Streptococci

Classification and identification of oral or viridans streptococci has long been regarded as a difficult area of streptococcal taxonomy (Hardie and Marsh, 1978b). Early attempts at classification on the basis of hemolysis and the possession of group-specific polysaccharide (Lancefield) antigens, which proved so useful with some streptococci (Lancefield, 1933), were unsuccessful (Lancefield, 1925a; Lancefield, 1925b). Subsequent efforts to classify the viridans streptococci by serological methods often resulted in the formation of groups exhibiting considerable biochemical heterogeneity and did not produce practically useful schemes (Solowey, 1942; Selbie et al., 1949; Williamson, 1964; Ball, 1985).

The use of biochemical and physiological tests in the division of the oral streptococci proved more successful. From the earliest applications of this approach (Gordon, 1905; Andrewes and Horder, 1906) to later numerical taxonomic studies (Carlsson, 1968; Colman, 1968; Hardie et al., 1982; Bridge and Sneath, 1983), the phenotypic characterization of strains formed the basis of the majority of taxonomic studies of these streptococci (for reviews of the literature, see Hardie and Bowden, 1976a; Hardie and Marsh, 1978b, and Coykendall, 1989a). Significant advances were achieved by combining data from cell wall

analysis (Colman and Williams, 1965), genetic transformation experiments (Colman, 1969), and a large numerical taxonomic investigation (Colman, 1968). From these studies, six distinct species were recognized among the viridans streptococci: *S. salivarius*, *S. mitior*, *S. milleri*, *S. sanguis*, *S. mutans* and *S. pneumoniae* (Colman and Williams, 1972; Colman, 1976).

During the 1960s and 1970s a large number of publications appeared on the taxonomy of the oral streptococci, but there were some disagreements regarding the proposed classification schemes (e.g., Carlsson, 1968; Colman and Williams, 1972; Hardie and Bowden, 1976a) and the nomenclatural systems used (Colman and Williams, 1972; Facklam, 1977). The increased use of genotypic criteria (including G+C content and extent of genomic sequence homology) followed the early transformation experiments of Colman (1969). Initially, these studies were directed toward resolving the heterogeneity observed within *Streptococcus mutans* (Coykendall, 1977), but eventually the techniques, particularly the estimation of whole genomic sequence homology by DNA-DNA hybridization, were used to clarify the taxonomy of the majority of the oral streptococci and provide a more reliable classification on which identification schemes could be based. Organisms for which these genetic approaches have been used include *S. mutans* and "mutans-like" streptococci (Coykendall et al., 1976; Coykendall, 1977; Coykendall, 1983; Beighton et al., 1984; Schleifer et al., 1984b; Whiley et al., 1988b; Zhu et al., 2000), *S. salivarius* and close relatives (Kilpper-Bälz et al., 1982; Farrow and Collins, 1984a; Coykendall and Gustafson, 1985; Whiley and Hardie, 1988a), the *S. milleri* group (Welborn et al., 1983; Farrow and Collins, 1984b; Kilpper-Bälz et al., 1984; Ezaki et al., 1986; Coykendall et al., 1987; Knight and Shlaes, 1988; Whiley and Hardie, 1989; Whiley et al. 1997; Whiley et al. 1999; Jacobs et al., 2000b), and *S. sanguis*, *S. oralis* ("*S. mitior*"), *S. mitis* and closely related taxa (Coykendall and Specht, 1975; Coykendall and Munzenmaier, 1978; Welborn et al., 1983; Kilpper-Bälz et al., 1985; Schmidhuber et al., 1987; Kawamura et al., 1998; Kawamura et al., 2000).

Other chemotaxonomic and molecular techniques have been applied to the study of oral streptococci (Schleifer and Kilpper-Bälz, 1987). These include cell wall (peptidoglycan) analyses (Schleifer and Kandler, 1972), multilocus enzyme electrophoresis (Gilmour et al., 1987), DNA-rRNA hybridization (Schleifer et al., 1984b; Kilpper-Bälz, 1985), and 16S rRNA sequence analyses (Bentley, et al., 1991; Kawamura et al., 1995a; Kawamura et al., 1995b; Ludwig et al., 1985). These approaches have shed light on the natural relationships among strepto-

coccal species (including the oral streptococcal species [which have been assigned to four phylogenetic units or species groups]), led to the division of the original genus *Streptococcus* into three genera: *Streptococcus*, *Enterococcus* and *Lactococcus* (Schleifer and Kilpper-Bälz, 1984a; Schleifer et al., 1985), and have been instrumental in describing several close relatives of the genus *Streptococcus* sensu stricto (Facklam and Elliot, 1995).

A brief outline of each of the recognized species of oral streptococci is given below, together with identification tables.

Mutans Group

Although the species *Streptococcus mutans* was not included in the eighth edition of *Bergey's Manual of Systematic Bacteriology* (Buchanan and Gibbons, 1974), subsequent taxonomic developments established a group that now consists of six species: *S. mutans*, *S. sobrinus*, *S. cricetus*, *S. rattus*, *S. macacae* and *S. downei*. A seventh species, *S. orisratti* has been described as a member of the mutans group by 16S rDNA sequence analysis, although it is most similar to *S. suis* by biochemical testing. An eighth species *S. ferus* is usually included alongside the other members of the mutans group, although this relationship has not been formally demonstrated and the true taxonomic position of this species has yet to be determined.

The streptococci originally designated *S. mutans* were isolated from carious human teeth by Clarke (1924). Despite Clarke's observations and the reported isolation of *S. mutans* from a patient with bacterial endocarditis (Abercrombie and Scott, 1928), little attention was directed towards this species until the early 1960s. The demonstration that caries could be experimentally induced and transmitted in animals (Fitzgerald and Keyes, 1960; Keyes, 1960), and that similar caries-inducing streptococci were present in human plaque (Zinner et al., 1965; Krasse, 1966), stimulated renewed interest in strains resembling *S. mutans*. The biochemical characteristics of this species enabled several authors to recognize clusters of strains to which it corresponded (Carlsson, 1968; Colman and Williams, 1972; Drucker and Melville, 1971). However, in contrast to the initially perceived phenotypic homogeneity of these strains, subsequent studies showed considerable heterogeneity among the mutans-like streptococci. Eight serovars, designated "a" through "h," have been demonstrated (Bratthall, 1970; Perch et al., 1974; Beighton et al., 1981), and variation on the basis of biochemical biotypes, cell walls (Hardie and Bowden, 1974a), electrophoretic separation of membrane proteins, comparative enzymes, intra-

cellular proteins, and whole-cell-derived proteins has been fully documented (see Hamada and Slade, 1980, and Hamada et al., 1986 for reviews of the literature).

The division of *S. mutans* strains into seven distinct species designated *S. mutans*, *S. sobrinus*, *S. rattus*, *S. cricetus*, *S. ferus*, *S. macacae* and *S. downei* resulted from a series of genotypic studies (Coykendall, 1974a; Coykendall, 1977; Coykendall, 1983; Coykendall et al., 1974b; Coykendall et al., 1976; Beighton et al., 1984; Whiley et al., 1988b). More recent studies have revealed a further species (proposed as *S. orisratti*; Zhu et al., 2000), which has *S. rattus* as its closest relative by 16S rDNA sequence analysis but which phenotypically most closely resembles *S. suis*. At best, this species can only be tentatively regarded as a member of this species group, with the results of DNA reassociation experiments giving no clear indication of an affiliation to any species group in particular. The taxonomic position of another of the species placed within the mutans group, *S. ferus*, is somewhat uncertain because this species is included on the basis of DNA similarity (Schleifer et al., 1984b) but appears to be more closely related to the mitis group by multilocus enzyme electrophoresis (Gilmour et al., 1987). The characteristics of the currently recognized species within the mutans group including *S. orisratti* and *S. ferus* are shown in Table 2.

Salivarius Group

The name *Streptococcus salivarius* was originally given by Andrewes and Horder (1906) to a relatively easily recognized streptococcus that was common in human saliva, present in the intestine, and also isolated occasionally from patients with endocarditis, terminal septicemia, and peritonitis. These streptococci characteristically produced short chains in broth, caused clotting of milk, reduced neutral red, produced acid from sucrose, lactose and raffinose (usually), rarely fermented inulin, never fermented mannitol, and were unable to grow on gelatin at 20°C.

Later workers tended to use fewer tests in assigning strains to *S. salivarius*, thus obscuring the distinction between this and the much more heterogeneous, *S. mitis*. Unfortunately many nonhemolytic streptococci were allocated to the poorly defined species *S. mitis*, and this led to considerable confusion.

Further characterization of *Streptococcus salivarius* on the basis of physiological properties (Sherman et al., 1943), extracellular polysaccharide (fructan/levan) production from sucrose (Niven et al., 1941), nutritional requirements (Niven and Smiley, 1942), and serology (Sherman et al., 1943; Farmer, 1953; Williams, 1956;

Montague and Knox, 1968) enabled this species to be identified relatively easily in subsequent taxonomic studies (Carlsson, 1968; Colman and Williams, 1972; Facklam, 1977; Hardie et al., 1982; Bridge and Sneath, 1983). Strains of *S. salivarius* are typically 1) nonhemolytic (although some β-hemolytic strains have been described; Saunders and Ball, 1980), 2) produce acid from inulin, lactose, raffinose, salicin and trehalose but not from mannitol, sorbitol or melibiose, 3) can hydrolyze esculin but not arginine, and 4) produce acetoin, and frequently urease. Most strains produce levan as an extracellular polysaccharide from sucrose, and they may react with Lancefield group K antiserum.

DNA-DNA hybridization experiments have demonstrated a close relationship between strains of *S. salivarius* and *S. thermophilus* (Kilpper-Bälz et al., 1982), and it was later proposed that these be redesignated *S. salivarius* subsp. *salivarius* and *S. salivarius* subsp. *thermophilus*, respectively (Farrow and Collins, 1984a). However, more extensive hybridization studies have shown that the actual level of DNA similarity shared between these two, albeit closely related, species and also with the newly described *S. vestibularis* does warrant separate species status for these organisms (Schleifer and Kilpper-Bälz, 1987; Whiley and Hardie, 1988a).

Streptococcus vestibularis (Whiley and Hardie, 1988a) is the name given to a group of α-hemolytic streptococci that had been isolated mainly from the vestibular mucosa of the human mouth and included several unidentified strains from an earlier numerical taxonomic study (Carlsson, 1968). These strains were distinguished by being able to 1) produce acid from lactose, salicin, and cellobiose but not mannitol, sorbitol, inulin, or raffinose, 2) hydrolyze starch and esculin but not arginine, 3) produce hydrogen peroxide, urease, and usually acetoin, but not extracellular polysaccharide from sucrose.

Chemotaxonomic data indicated a close relationship with *S. salivarius* from the presence of eicosenoic (C20:1) acids by capillary gas-liquid chromatography and from whole-cell-derived polypeptide patterns by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). DNA-DNA hybridization studies confirmed that *S. vestibularis* strains represented one of a group of three closely related species that also included *S. salivarius* and *S. thermophilus*.

The distinguishing characteristics of *S. salivarius*, *S. vestibularis* and *S. thermophilus* are shown in Table 3.

Anginosus Group

The background of the taxonomy and nomenclature of the streptococci within this group is

Table 2. Biochemical characteristics of species within the mutans group.

Test	S. mutans	S. sobrinus	S. cricetus	S. rattus	S. macacae	S. downei	S. ferus	S. orisratti
Enzyme activity								
β-D-Fucosidase	–	–	ND	ND	ND	ND	ND	ND
β-*N*-Acetyl-gal	–	–	ND	ND	ND	ND	ND	ND
Neuraminidase	–	–	ND	ND	ND	ND	ND	ND
α-L-Fucosidase	–	–	ND	ND	ND	ND	ND	ND
β-*N*-Acetyl-glu	–	–	ND	ND	ND	ND	ND	ND
α-D-Glucosidase	+	+	ND	ND	ND	ND	ND	ND
β-D-Glucosidase	+	–	ND	ND	ND	ND	ND	ND
α-Arabinosidase	–	–	ND	ND	ND	ND	ND	ND
α-D-Galactosidase	+	–	ND	ND	ND	ND	ND	–
β-D-Galactosidase	–	–	ND	ND	ND	ND	ND	–
Acid produced from								
Amygdalin	+/–	–	+	+	+	ND	+	+
Inulin	+	–	+	+	–	+	+	+
Mannitol	+	+	+	+	+	+	+	–
N-acetyl-glu	+	–	+	+	+	ND	+	ND
Raffinose	+	d	+	+	+	–	–	+
Sorbitol	+	d	+	+	+	–	+	–
Arbutin	+[b]	–	+	+	ND	ND	+	+
Lactose	+	+/–	+/–	+/–	ND	+	+	+
Melibiose	+	d	+	+	–	–	–	+
Cellobiose	+	+/–	+	+	+	–	+	+
Maltose	+	+	+	+	+	+	+	+
Melezitose	ND	ND	ND	ND	ND	ND	ND	–
Ribose	–	–	–	–	–	ND	–	–
Trehalose	+	+	+/–	+	+	+	+	+
Hydrolysis of								
Arginine	–	–	–	+	–	–	–	–
Esculin	+	d	d	+	+	–	+	+
Production of								
Acetoin (VP)	+	+	+	+	+	+	+	–
PAL	–	–	ND	ND	ND	–	ND	–
ecp	+	+	+	+	+	+	+	–
H₂O₂	–	+	–	–	–	–	–	
Urease	–	–	–	–	ND	ND	–	ND
Resistance to bacitracin	+	+	–	+	–	–	–	ND
Serotype[a]	c, e and f	d and g	a	b	c	h	c	A

Symbols: +, ≥90% of strains are positive; +/–, 50–89% of strains are positive; –/+, 11–49% of strains are positive and –, ≤10% of strains are positive.

Abbreviations: ND, not determined; β-*N*-acetyl-gal, detection of β-*N*-acetyl-galactosaminidase activity; β-*N*-acetyl-glu, detection of β-*N*-acetyl-glucosaminidase activity; *N*-acetyl-glu, production of acid from *N*-acetyl-glucosamine; PAL, alkaline phosphatase; ecp, extracellular polysaccharide production from sucrose; and H₂O₂, production of hydrogen peroxide.

[a]Serotypes: a–h (see references below) and A, Lancefield group A.

Based on data from: Bratthall (1970); Parch et al. (1974); Beighton et al. (1981); Kral and Daneo-Moore (1981); Costendall (1983); Hardie (1986b); Whiley et al. (1988); Whiley and Beighton (1998); and Zhu et al. (2000).

confused (Coykendall et al., 1987). Much of our current understanding of these streptococci rests on the work of Colman and Williams (1972) who recognized an overall similarity among strains variously referred to in the literature as "*Streptococcus MG*" (Mirick et al., 1944), the hemolytic and nonhemolytic streptococci of Lancefield group F (Ottens and Winkler, 1962), the minute-colony-forming streptococci of Lancefield groups F and G (Long and Bliss, 1934; Bliss, 1937), and those streptococci given the name "*S. milleri*" by Guthof (1956). Guthof had first proposed the name *S. milleri* for a group of nonhemolytic streptococci isolated from puru-

lent oral infections: 1) able to hydrolyze esculin and arginine, 2) grow on 40% bile agar and at 45°C, but 3) unable to ferment mannitol or sorbitol. Facklam (1977) also noted a close similarity among several species previously described by different authors as *S. anginosus* (Andrewes and Horder, 1906; Deibel and Seeley, 1974), *S. constellatus* (Holdeman and Moore, 1974), *S. intermedius* (Holdeman and Moore, 1974), and *Streptococcus MG* (Mirick et al., 1944). Rather than include all these together within *S. milleri* as Colman and Williams had suggested, Facklam divided them into two groups, designated *S. MG-intermedius* (lactose fermenters) and *S.*

Table 3. Biochemical characteristics of *S. salivarius* and closely related species.

Test	*S. salivarius*	*S. vestibularis*	*S. thermophilus*
Enzyme activity			
β-D-Fucosidase	+/–[a]	–	ND
β-*N*-Acetyl-gal	–	–	ND
Neuraminidase	–	–	ND
α-L-Fucosidase	–	–	–
β-*N*-Acetyl-glu	–	–	–
α-D-Glucosidase	–/+[a]	+/–	–
β-D-Glucosidase	+/–	–	–
α-Arabinosidase	+	+	ND
α-Galactosidase	–/+	–	–
β-Galactosidase	+/–	+	+
Acid from			
Amygdalin	+/–[b]	+/–	–
Inulin	+/–	–	–
Mannitol	–	–	–
N-Acetyl-glu	+[b]	+[b]	–
Raffinose	+/–[b]	–	–/+
Sorbitol	–	–	–
Arbutin	+	–/+	V[c]
Lactose	+	+/–	+
Melibiose	–	–	V[c]
Cellobiose	+	+/–	–
Maltose	+	+	–
Melezitose	–	–	V[c]
Ribose	–	–	V[c]
Trehalose	+/–	–/+	–
Hydrolysis of			
Arginine	–	–	–
Esculin	+	+	–
Production of			
Acetoin (Voges-Proskauer)	+/–	+/–	+
PAL[a]	+/–	–	–
ecp[a]	+	–	–
H₂O₂	–	+	–
Urease	–/+	+	–
Growth at 45°C	–	–	+

Symbols: See footnote in Table 2.

Abbreviations: See footnote in Table 2.

[a]The proportion of strains reported as giving positive reaction may vary depending on the method used.

[b]The proportion of strains reported as giving positive reaction varies between studies.

[c]Reported as variable.

Based on data from: Whiley and Hardie (1988); Kilian et al. (1989a); Schleifer et al. (199l); and Beighton et al. (1991).

anginosus-constellatus (lactose nonfermenters). In practice, most British and other European microbiologists tended to include all such strains within *S. milleri* according to the Colman and Williams' approach, while many in the United States followed Facklam's system. In an attempt to reduce the confusion caused by the use of two conflicting nomenclatures, Facklam (1984) suggested that nonhemolytic strains should continue to be divided on the basis of lactose fermentation into *S. constellatus* (lactose nonfermenters) and *S. intermedius* (lactose fermenters), with β-hemolytic strains (in possession of a Lancefield group antigen A, C, F or G, or remaining ungroupable) designated "*S. anginosus*."

In addition to nomenclatural problems, results from taxonomic studies on the anginosus group have been contradictory. While several investigations have indicated that these strains are closely related, sharing a high degree of phenotypic similarity (Mejaré, 1975a; Mejaré and Edwardsson, 1975b; Lütticken et al., 1978; Labbe et al., 1985; Coykendall et al., 1987; French et al., 1989; Vandamme et al., 1998), others have demonstrated heterogeneity on the basis of long-chain fatty acid analyses (Drucker and Lee, 1981; Cookson et al., 1989), serological data (Colman and Williams, 1972; Lütticken et al., 1978; Yakushiji et al., 1988), G+C content (Drucker and Lee, 1983), fermentation patterns (Poole and Wilson, 1979;

Drucker and Lee, 1981; Ruoff and Kunz, 1982; Winstanley et al., 1992), and pyrolysis mass spectrometry (Winstanley et al., 1992). Conflicting data have also been obtained from the several published DNA-DNA hybridization studies. Some have concluded that strains within the anginosus group should be regarded as belonging to a single species (Welborn et al., 1983; Farrow and Collins, 1984b; Ezaki et al., 1986; Coykendall et al., 1987), whereas others have detected several centers of taxonomic variation among these streptococci (Kilpper-Bälz et al., 1984; Knight and Shlaes, 1988; Whiley and Hardie, 1989; Kitada et al., 1992; Whiley et al., 1997; Whiley et al., 1999). Some of these differences may have been due to the respective stringencies of the methods employed (Coykendall et al., 1987; Knight and Shlaes, 1988; Whiley and Hardie, 1989). In some cases, the selection of strains for study on the basis of the type of hemolysis produced on blood agar and on lactose fermentation may have produced data that were unrepresentative of the species group as a whole (Ezaki et al., 1986; Knight and Shlaes, 1988). Studies using DNA-DNA hybridization and phenotypic tests confirmed the presence of three distinct, albeit closely related, taxa that include the reference strains of *S. constellatus* (National Collection of Dairy Organisms [NCDO 2226]), *S. intermedius* (NCDO 2227) and *S. anginosus* (NCTC 10713), respectively (Whiley and Hardie, 1989; Whiley et al., 1990b). Also, DNA reassociation studies further revealed heterogeneity within both *S. anginosus* and *S. constellatus*, particularly those strains exhibiting a β-hemolytic, Lancefield group C phenotype (Whiley et al., 1997). Subsequent research has led to the proposal that *S. constellatus* constitutes two subspecies: *S. constellatus* subsp. *constellatus* (isolated from a relatively broad clinical background) and *S. constellatus* subsp. *pharyngis* (exhibiting a predilection for sites within the human throat and from cases of pharyngitis) for the majority of β-hemolytic, Lancefield group C strains belonging to this species (Whiley et al., 1999). Two DNA similarity groups were also detected within *S. anginosus*, with most β-hemolytic, Lancefield group C strains tested comprising one of these groups. However, the lack of discriminating biochemical tests for identification precluded any formal taxonomic proposals regarding further subdivision of *S. anginosus*.

Alternative sources of genotypic evidence supports the recognition of *S. anginosus*, *S. constellatus* and *S. intermedius* within the anginosus group (Bentley et al., 1991; Kawamura et al., 1995a; Poyart et al., 1998) and indicates that further centers of taxonomic variation exist (Whiley et al., 1995; Jacobs et al., 1996; Jacobs et al., 2000b; Jacobs et al., 2000c). The usefulness of the current classification of strains within the anginosus group into three species is exemplified by the observation of a novel, human specific virulence factor named "intermedilysin," that is only coded for and expressed by all strains of *S. intermedius* so far examined and appears to be absent from *S. anginosus* and *S. constellatus* (Nagamune et al., 2000).

Differential characteristics of the three species within the anginosus group are listed in Table 4.

Mitis Group

Kawamura et al. (Kawamura et al., 1995a; Kawamura et al., 1995b) gave the name "mitis group" to the phylogenetic unit that currently includes: *S. mitis*, *S. sanguis*, *S. gordonii*, *S. parasanguis*, *S. crista*, *S. pneumoniae*, *S. oralis*, *S. peroris*, *S. infantis* and *S. australis*. These correspond to the "*Streptococcus oralis* group" described by previous authors (Schleifer and Kilpper-Bälz, 1987; Bentley et al., 1991), although the latter authors also included the anginosus group members *S. anginosus*, *S. intermedius* and *S. constellatus*. Besides the named species, there is at least one further center of taxonomic variation, or genospecies, that has been revealed by DNA-DNA reassociation (Kawamura et al., 2000). The history of the classification and nomenclature of the species described here is convoluted and confusing and has been described in detail elsewhere (Whiley and Beighton, 1998).

STREPTOCOCCUS MITIS *Streptococcus mitis* was the name originally used by Andrewes and Horder (1906) to describe a short-chained saprophytic streptococcus (mainly found in human saliva and feces) that grows well at 20°C on gelatin, does not clot milk but often reduces neutral red, nearly always ferments lactose and sucrose and sometimes the glucosides salicin and coniferin.

Owing to the small number of tests later used to identify strains as *S. mitis* (e.g., Holman, 1916), this species was often poorly differentiated from *S. salivarius*. Sherman (1937) emphasized the unsatisfactory description of *S. mitis* in his review and used the term "S. Mitis Group" for what had become a rather ill-defined and heterogeneous collection of strains that were characterized mainly on the basis of negative characters. They were described as producing marked greening (α-hemolysis), being unable to ferment arabinose, glycerol, inulin, mannitol, sorbitol and xylose, and unable to hydrolyze sodium hippurate or produce polysaccharide from sucrose. Most strains fermented salicin, and were sometimes able to ferment raffinose, hydrolyze arginine and esculin, and grow at 45°C, but were seldom able to ferment trehalose or grow on 10% bile agar. The production of ammonia from

Table 4. Differential characteristics of strains of *S. anginosus*, *S. constellatus* and *S. intermedius*.

| | | S. constellatus | | |
| | | subsp. | subsp. | |
Test	*S. anginosus*	*constellatus*	*pharyngis*	*S. intermedius*
Enzyme activity				
β-D-Fucosidase	−	−	+	+
β-*N*-Acetyl-gal[a]	−	−	+	+
Neuraminidase	−	−	−	+
α-L-Fucosidase	−	−	−	−
β-*N*-Acetyl-glu[a]	−	−	+	+
α-D-Glucosidase[a,b]	−/+	+	+	+
β-D-Glucosidase[a,b]	+	−	+	−/+
α-Arabinosidase	−	−	−	−
α-D-Galactosidase[b]	−/+	−	−	−
β-D-Galactosidase[a,b]	−/+	−/+	+	+
Acid from				
Amygdalin	+	−/+	+	+/−
Iinulin	−	−	−	−
Mannitol	−	−	−	−
Raffinose	−/+	−	−	−
Sorbitol	−	−	−	−
Arbutin	+	+	+	+
Lactose	+	+/−	+	+
Melibiose	−/+	−/+	−	−/+
Hydrolysis of				
Arginine	+	+	+	+
Esculin	+	+/−[c]	+[c]	+
Production of				
Acetoin (VP)	+	+	+	+
Urease	−	−	−	−
Hyaluronidase	−	+	+/−	+
Hemolysis	α, β and γ	α, β and γ	β	α, γ and β
Lancefield groups	−, A, C, F and G	−, A, C, F and G	C	−, C and F

Symbols: See footnote in Table 2.

Abbreviations: See footnote in Table 2; and VP, Voges-Proskauer.

[a]The proportion of strains reported as giving positive reaction varies between studies.

[b]The proportion of strains reported as giving positive reaction may vary depending on the method used.

[c]Weak or slow reactions given by some strains.

Based on data from: Kilpper-Bälz et al. (1984); Whiley et al. (1990b, 1999); and Jacobs et al. (1995).

arginine by some strains was taken to indicate that more than one species was present in this group. Streptococci identified as *S. mitis* according to the description of Sherman et al. (1943) were isolated in a number of studies from the human throat, the oral cavity, and from blood, including blood of patients with subacute bacterial endocarditis (Loewe et al., 1946; Niven and White, 1946; Farmer, 1953; Morris, 1954; Carlsson, 1967; Guggenheim, 1968). Failure to demonstrate a group antigen (Sherman et al., 1943) was followed by studies that showed the mitis group to be serologically heterogeneous (Farmer 1953; Williamson, 1964). Some studies also reported the production of dextran from strains considered to be *S. mitis* (Hehre and Neill, 1946; Guggenheim, 1968).

Despite the emphasis placed in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (Deibel and Seeley, 1974) on the poor

definition of this species, the name *S. mitis* was included in the Approved Lists of Bacterial Names (Skerman et al., 1980), with strain NCTC 3165 assigned as type strain. Unfortunately, this strain was inappropriate since it more closely resembled *S. sanguis* (*S. sanguis* biotype I). Streptococcal strains considered by many bacteriologists to belong to *S. mitis* were those otherwise referred to as *S. sanguis* biotype II or "*S. mitior*" (Colman and Williams, 1972; Rosan, 1973; Cole et al., 1976; Kilian et al., 1986). DNA-DNA hybridization studies (Welborn et al., 1983; Kilpper-Bälz et al., 1985; Schleifer and Kilpper-Bälz, 1987; Schmidhuber et al., 1987) have clearly demonstrated that strain NCTC 3165 belongs to a distinct taxon for which the name "*Streptococcus gordonii*" has been proposed (Kilian et al., 1989b).

Formal proposals for the rejection of strain NCTC 3165 as the type species of *S. mitis*

(Coykendall, 1989b; Kilian et al., 1989a) were made and its replacement with the new type strain NS51 (= NCTC 12261; Kilian et al., 1989a) was subsequently proposed. These authors also proposed to assign to the species *S. mitis* those streptococci that share the same peptidoglycan type (lysine direct), lack both glycerol teichoic acid and significant amounts of rhamnose, but which contain a ribitol teichoic acid in their cell walls (Colman and Williams, 1965; Colman and Williams, 1972; Rosan, 1976; Kilpper-Bälz et al., 1985). These strains sometimes hydrolyze arginine, do not produce extracellular polysaccharide from sucrose, and produce IgA$_1$ protease less frequently than do strains of *S. oralis*. Several of the strains included by Kilian et al. (1989b) in their description of *S. mitis*, including the type strain NS51, previously formed a distinct DNA similarity group (Coykendall and Munzenmaier, 1978) sharing 30–35% DNA similarity with strains ultimately shown to belong to *S. oralis* (Bridge and Sneath, 1982; Kilpper-Bälz et al., 1985). Some of the distinguishing characteristics of *S. mitis* as now recognized are listed in Table 5, although the extension of this species to two biotypes (Kilian et al., 1989b) has resulted in the inclusion of strains of disparate taxonomic affiliation within *S. mitis* biovar 2 (Kikuchi et al., 1995; Vandamme et al., 1998; De Gheldre et al., 1999)

Streptococcus sanguis The taxonomic history of *S. sanguis* provides an excellent example of the progress that resulted from a shift away from a predominantly serological approach to the use of biochemical and physiological data, cell wall studies, and finally to the application of genotypic criteria.

Streptococcus sanguis was the name given by White and Niven (1946) to the α-hemolytic, dextran-forming streptococci isolated from the blood clots and heart vegetations of patients with bacterial endocarditis. It had previously been referred to as "*Streptococcus s. b. e.*" (Loewe et al., 1946). The early literature was largely concerned with the serological and antigenic analyses of strains variously called "*Streptococcus s. b. e,*" "*S. sanguis,*" and the "Lancefield group H streptococci." The latter strains (described by Hare, 1935) were originally isolated from human throats, but unfortunately the strain used to raise the original group H antiserum was not recorded. Later investigators reporting the presence of H antigen in some strains of *S. sanguis* used different strains to raise their group H antisera (Dodd, 1949; Porterfield, 1950; Farmer, 1954), with the result that the identity of the group H antigen and the taxonomic position of these strains remained the subject of

much confusion (Cole et al., 1976; Hardie and Bowden, 1976b). Further serological analysis of strains identified as *S. sanguis* led to the description of antigens a–e, of which antigen a, characterized as a glycerol teichoic acid, was considered to be the group H antigen (Rosan 1973; Rosan 1976; Rosan and Argenbright, 1982). However, several of the strains examined in Rosan's studies fall into the newly described species *S. gordonii* (Kilian et al., 1989b). Thus it appears that there is a considerable degree of overlap of these antigens, including the group H antigen, among different taxa.

Although the name "*S. sanguis*" had originally been used to describe dextran-producing strains, Colman and Williams (1972) considered that both dextran-positive and dextran-negative strains should be included within this species. *Streptococcus sanguis* was characterized as being: usually α-hemolytic, producing hydrogen peroxide, able to hydrolyze arginine and esculin, able to produce acid from trehalose and salicin, infrequently fermenting raffinose, but unable to produce acid from mannitol, sorbitol, arabinose and glycerol. These authors excluded those strains previously designated as "*S. sanguis* serotype II" (Washburn et al., 1946), which lacked rhamnose in their cell walls and failed to hydrolyze arginine or esculin, preferring to name these isolates "*S. mitior*" (Schottmüller, 1903).

An alternative nomenclature was proposed by Facklam (1977) who suggested dividing those strains resembling "*S. mitior*" of Colman and Williams (1972) into two groups on the basis of raffinose fermentation: *S. sanguis* biotype II (raffinose fermenters) and *S. mitis* (raffinose nonfermenters). In Facklam's system, strains of *S. sanguis*, as defined by Colman and Williams, were designated "*S. sanguis* biotype I." The use of different nomenclatural systems for these streptococci gave rise to considerable confusion in the literature.

DNA-based studies (Coykendall and Specht, 1975; Coykendall and Munzenmaier, 1978; Welborn et al., 1983; Schmidhuber et al., 1987) revealed the existence of four DNA similarity groups within strains of *S. sanguis* (Colman and Williams, 1972; Colman, 1976) or *S. sanguis* biotype I (Facklam, 1977). Such studies also unequivocally separated these streptococci from strains corresponding to "*S. mitior*" (Colman and Williams, 1972; Colman, 1976) or *S. sanguis* biotype II/*S. mitis* (Facklam, 1977). Two of these four genetic groups are represented by the validly published species *S. sanguis* (type strain NCTC 7863 = ATCC 10556) and *S. gordonii* (type strain NCTC 12261; Kilian et al., 1989b). Within the latter group is included strain NCTC 3165, which was previously given as the type

Table 5. Biochemical characteristics of the Mitis Group.

Test	S. sanguis	S. parasanguis	S. gordonii	S. crista	S. oralis	S. mitis	S. peroris	S. infantis	S. australis
Enzyme activity									
β-D-Fucosidase	+/–[a]	–/+	–	–	–[a]	–[a]	–	+/–	ND
β-N-Acetyl-gal[c]	–	+	+/–	+	+	–	ND	ND	+
Neuraminidase	–	–	–	–	+	–/+	ND	ND	–
α-L-Fucosidase	–	–/+	+	+	–	–	ND	ND	ND
β-N-Acetyl-glu[d]	–/+[a]	+	+	+	+	–[a]	–	+/–	–[b]
α-D-Glucosidase[e]	–	+	–/+[a]	–	+	+[a]	ND	ND	ND
β-D-Glucosidase[e]	+/–	–/+	+	–	–	–[a]	–	–	–
α-Arabinosidase	–	–	–	–	–	–	ND	ND	ND
α-D-Galactosidase[e]	+/–	+	–/+	–	–/+	+/–	–	–	–
β-D-Galactosidase[e]	–/+[a]	+	–/+[a]	–/+	+/–	–/+[a]	–	+	+[b]
Acid from									
Amygdalin	–	–/+	+	–	–	–	–	–	ND
Inulin	–/+	–	+	–	–	–	–	–/+	ND
Mannitol	–	–	–	–	–	–	–	–	–
N-Acetyl-glu[f]	+	+	+	+	+	+	ND	ND	ND
Raffinose	+/–	+/–	–/+	–	+/–	+	–	–	–
Sorbitol	–/+	–	–	–	–	–	–	–	–
Arbutin	+/–	–/+	+	+	+	–	–	+	ND
Lactose	+	+	+	+/–	+	+/–	+	+	+
Melibiose	+/–	+/–	–/+	–	–/+	+	–	–	–
Tagatose	ND	+/–	–/+	+/–	–/+	–	–	+	–
Pullulan	+	–	–	+/–	+	+/–	–	–/+	+
Hydrolysis of									
Arginine	+	+	+	+/–	–	–[a]	–	–	+
Esculin	+/–	–/+	+	–	–/+	–	–	–	ND
Production of									
Acetoin (VP)	–	–	–	–	–	–	–	–	–
Urease	–	–	–	–	–	–	–	–	–
Hyaluronidase	–	–	+	–	–	–	ND	ND	ND
Alkaline phosphatase	ND	+	+	–	+/–	+/–	–/+	–	+[b]
ecp	+	–	+	V[c]	+/–	–	ND	ND	–
IgA protease	+	ND	–	ND	+	–/+	ND	ND	ND

Symbols: See footnote in Table 2.
Abbreviations: See footnote in Table 2; and VP, Voges-Proskauer.
[a]The proportion of strains reported as giving positive reaction varies between studies.
[b]The proportion of strains reported as giving positive reactions may vary depending on the method used.
[c]Reported as variable.
Based on data from: Kilian et al. (1989a); Beighton et al. (1991); Hardie and Whiley (1995); Kikuchi et al. (1995); Kawamura et al. (1998); and Willcox et al. (2001).

Table 6. Biovars (biotypes) within the "mitis group."

Test	*S. sanguis* biovars[a]				*S. sanguis* biotypes[b]			*S. gordonii* biovars			*S. mitis* biovars	
	1	2	3	4	1	2	3	1	2	3	1	2
Enzyme activity												
α-L-Fucosidase	−	−	−	−	−	−	−	+	+	+	−	−
β-D-Fucosidase	+	−	−	−	−	+	+/−	−	−	−	−/+	+/−
α-D-Glucosidase	−	−	−	−	−	−	−	+/−	+	+	+/−	+
β-D-Glucosidase	+	+	+	+	−/+	+	−/+	+	+	+	−	+
α-D-Galactosidase	+	+	+	−	+/−	+	−	+	+/−	−	+/−	+
β-D-Galactosidase	+	+	+	+	−	−/+	+	+	+	+	+	+
β-*N*-Acetyl-glu	+	−	+	−	−	−/+	+	+	+	+	+	+
Neuraminidase	−	−	−	−	−	−	−	−	−	−	−/+	+/−
Acid from												
Amygdalin	−	−	−	−/+	−	+	−	+	+	+	−	−
Arbutin	+/−	+	+	+	+/−	−/+	+	+	+	+	−	−
Inulin	+	+	+	+/−	−/+	−	+	+	+/−	+	−	−
Melibiose	+	−	+/−	−	+	+/−	−	+	−	−	−/+	+
Raffinose	+	−	+/−	−	+	+	−	+	−	−	+/−	+
Sorbitol	−	−	+/−	−	−/+	+/−	−	−	−	−	−	−/+
Trehalose	+	+	+	+	ND	ND	ND	+	+	+	−/+	−
Salicin	+	+	+	+	ND	ND	ND	+	+	+	−/+	−/+
Hydrolysis of												
Arginine	+	+	+	+	+	+	+	+	+	+	−	+
Esculin	+	−	+	+	+	+	−	+	+	+	−	−
Production of												
Alkaline phosphatase	−/+	−	−	−	ND	ND	ND	+	+	+	+/−	+/−
ecp	+	+	+	+	ND	ND	ND	+	+	+/−	−	−
IgA protease	+	+	+	+	ND	ND	ND	−	−	−	−/+	−

Symbols: See footnote in Table 2.
Abbreviations: See footnote in Table 2.
[a]Data from Kilian et al., 1989a.
[b]Data from Beighton et al., 1991.

strain of *S. mitis* in the Approved Lists of Bacterial Names (Skerman et al., 1980). Phenotypically, this strain did not closely match the original description of *S. mitis* and an alternative type strain was proposed (Coykendall, 1989b; Kilian et al., 1989a) as described above.

The third genetic group among the sanguis-like streptococci was shown to represent a new species and given the name "*Streptococcus parasanguis*" (Whiley et al., 1990a). The fourth known group, known for some time only as the "tufted fibril group" (Handley et al., 1985) formed a distinct species and was eventually given the name "*Streptococcus crista*" (Handley et al., 1991).

Differential characteristics of *S. sanguis* and related species are shown in Table 5.

The division, by some authors (Kilian et al., 1989b; Beighton et al., 1991), of *S. sanguis* and some other mitis group species into biotypes (biovars) is summarized in Table 6.

It is interesting to note that there are apparently three registered 16S rRNA gene sequences of the type strain of *S. sanguis* (accession numbers X53653, AF003928 and AB002524), which have 4–13 base differences within an area of approximately 1,300 bp (Kawamura et al., 1998). However, despite these differences, which are thought to have been due to technical problems leading to misinterpretation of sequence data, the position of *S. sanguis* as a member of the mitis group remains inviolate.

STREPTOCOCCUS GORDONII *Streptococcus gordonii* is the name given by Kilian et al. (1989b) to the group of strains that closely resemble *S. sanguis* and to which the former type strain of *S. mitis* (NCTC 3165) has been shown to belong in DNA-DNA hybridization studies (Coykendall and Specht, 1975; Schmidhuber et al., 1987).

The phenotypic similarity of these strains to *S. sanguis* stems from their ability to hydrolyze arginine and esculin and to produce extracellular polysaccharide from sucrose, but they differ in their ability to ferment amygdalin, and in possessing β-glucosaminidase, β-mannosidase, α-L-fucosidase, and a strong alkaline phosphatase activity (Kilian et al., 1989b). They also exhibit lower G+C content (38–39 mol%) compared to *S. sanguis* (40–43 mol%) and are able to bind salivary α-amylase (Douglas et al., 1990).

Although strain NCTC 3165 is now included in the species *S. gordonii*, it is not phenotypically typical since it lacks the ability to produce extracellular polysaccharide but does possess β-galactosidase activity. Not surprisingly, therefore, it has not been suggested as the type strain. Properties of *S. gordonii* are shown in Table 5.

Streptococcus parasanguis The name "*Streptococcus parasanguis*" was proposed for a group of strains isolated from clinical specimens (throats, blood and urine). On the basis of DNA hybridization studies, the relationship of α-hemolytic streptococci had been shown to be closer to *S. sanguis* than to other oral streptococci (Whiley et al., 1990a). Some of the strains included in this species had fallen into unnamed DNA similarity groups in previous studies, namely the MGH group, *S. intermedius* DNA similarity group III (Knight and Shlaes, 1988), and DNA similarity group IV (Whiley and Hardie, 1989).

Biochemical and physiological characteristics useful in differentiating strains of *S. parasanguis* from other oral streptococci are shown in Table 5.

Streptococcus crista An unnamed genetic group within strains resembling *S. sanguis* from human throats and mouths was reported on the basis of DNA-DNA hybridization experiments (Coykendall, 1989a). These strains were characterized by 1) the presence of tufts and fibrils on their cell walls (Handley et al., 1985), 2) the ability to hydrolyze arginine, but very weak ability or inability to hydrolyze esculin, 3) lack of acid production from raffinose, or 4) lack of alkaline phosphatase production. They were referred to variously in the literature as "*S. sanguis* I with tufts or fibrils" (Handley et al., 1985), the "CR group" (Douglas et al., 1990) and the "tufted fibril group" (Beighton et al., 1991). Subsequent DNA reassociation experiments demonstrated that these represented a distinct species and were given the name "*Streptococcus crista*" (Handley et al., 1991).

Streptococcus oralis *Streptococcus oralis* was the name given by Bridge and Sneath (Bridge and Sneath, 1982; Bridge and Sneath, 1983) to a group of isolates originally from the human oral cavity (Carlsson, 1967) that included strains corresponding to both *S. sanguis* and "*S. mitior*" (Colman and Williams, 1972).

In a later study of the cell wall and physiology of this species, also involving nucleic acid hybridization, those strains found to resemble *S. sanguis* were excluded, and an emended description of *S. oralis* was given (Kilpper-Bälz et al., 1985). Cell walls of the redefined species contained rib-

itol and choline, but lacked rhamnose, and possessed directly cross-linked peptidoglycan with lysine as the diamino acid. These studies also indicated that *S. oralis* strains were relatively closely related to strains of *S. pneumoniae*.

The valid publication of the description of *S. oralis* (Bridge and Sneath, 1982; Kilpper-Bälz et al., 1985) meant that this was the approved name for strains identified in the past as "*S. mitior*" (Colman and Williams, 1972), *S. sanguis* biotype II, or *S. mitis* (Facklam, 1977).

Some of the characters of *S. oralis* are shown in Table 5.

Streptococcus peroris *Streptococcus peroris* (39.8–40.5 mol% G+C) is one of two new α-hemolytic species (together with *Streptococcus infantis*) that have recently been added to the mitis group. This species was isolated from the human pharynx and teeth including the pharynges and teeth of patients with Kawasaki disease, although no data supporting an active involvement in this condition were presented (Kawamura et al., 1998). This species is grouped within the mitis group close to *S. gordonii* by 16S rRNA gene sequencing and close to *S. infantis* by DNA reassociation. Biochemically this species can be differentiated from the other mitis group members on the basis of predominantly negative results, according to the published identification scheme (Kawamura et al., 1998).

The biochemical characteristics of *S. peroris* are shown in Table 5.

Streptococcus infantis *Streptococcus infantis* (39.9–40.4 mol%G+C) was isolated from the same clinical sites as *S. peroris* and also included strains from patients with Kawasaki disease. This species is characterized by the ability to ferment tagatose, to produce β-galactosidase, and the frequent production of *N*-acetyl-β-glucosaminidase according to the published biochemical scheme (Kawamura et al., 1998).

The biochemical characteristics of *S. infantis* are shown in Table 5.

Streptococcus australis This species has been isolated from the saliva of healthy children (Willcox et al., 2001). The failure to isolate these streptococci from adults has led the authors to speculate that this species is an early colonizer of the oral cavity in childhood but is later displaced. Isolates were characteristically able to grow on high (up to 500 mM) NaCl- and KCl-containing media. DNA-DNA hybridization and 16S rRNA gene sequencing demonstrated these to constitute a new species within the mitis group.

The biochemical characteristics of *S. australis* are shown in Table 5.

Tufted Mitior Strains (*Streptococcus sanguis* Biotype II)

Carrying tufted fibrils on their surface, another group of oral streptococci that do not hydrolyze arginine or esculin were originally referred to as "(tufted) *Streptococcus sanguis* biotype II" or "tufted mitior" (Handley et al., 1985). By 16S rDNA sequence analysis, these streptococci have been shown to belong to the mitis group and share >95% overall genomic DNA sequence similarity (Kawamura et al., 2000). In a previous study, both biochemical testing and pyrolysis mass spectrometry were reported to have differentiated tufted *S. mitis* strains from other taxa (Magee et al., 1997). However, despite extensive testing for differential phenotypic characteristics, these streptococci could not be separated from *S. mitis* on biochemical grounds, thus remaining unidentifiable and therefore reported as a genospecies only (Kawamura et al., 2000). So far only three strains have been isolated and tested. The isolation of further strains will hopefully allow acquisition of a more accurate phenotypic profile of these streptococci, may reveal useful tests for their identification, and enable a species epithet to be proposed.

Identification of Oral Streptococci

Although biochemical test-based identification schemes that attempt to keep abreast of taxonomic developments have been reported for the oral streptococci, the addition of recently proposed species and as yet unnamed taxa (genospecies) means that a reliable, all-encompassing scheme is not available. The detection of a range of glycosidase activities using either chromogenic or fluorogenic substrates has improved the inherent resolving power of test schemes, and these have been incorporated into both in-house (Kilian et al., 1989b; Beighton et al., 1991) and commercial (Freney et al., 1992) formats, although additional tests may be required for confident identification (Douglas et al., 1990; Freney et al., 1992; Kikuchi et al., 1995). Difficult areas within the oral streptococci (in particular, the mitis and anginosus species groups) include as yet unnamed taxa. In a recent study evaluating commercial test kits, less than 79% accuracy (correct identification) was observed within the mitis group (Kawamura et al., 1999). In addition, commercial test kit databases may not include all currently recognized species, particularly those species infrequently isolated and for which few isolates are available for study (Kikuchi et al., 1995). Identification of oral streptococci is complicated further by the division of certain species into biotypes according to different criteria (Kilian et al., 1989b; Beighton et al., 1991). Kilian et al. described four biotypes within *S. sanguis*, three within *S. gordonii* and two within *S. mitis*, whereas Beighton et al., described only three biotypes for *S. sanguis* and did not subdivide *S. gordonii* or *S. mitis*. Subsequent studies using cellular protein patterns and genetic polymorphisms as criteria indicate that *S. mitis* biovar 2 (Kilian et al., 1989b) is a taxonomically heterogeneous group of strains that either group together with one of several different species within the mitis group or remain ungrouped (Vandamme et al., 1998; De Gheldre et al., 1999). The difficulties of identifying strains of *S. mitis*, in particular biotype 2, have been highlighted in a study where the disparate taxonomic positions of the strains were confirmed by whole genomic DNA-DNA hybridization (Kikuchi et al., 1995).

Alternative approaches to identifying oral streptococci have been applied. Recent studies include whole cell signatures derived from SDS-PAGE (Vandamme et al., 1998), pyrolysis mass spectrometry (Magee et al., 1997) and Fourier transform infrared spectroscopy (Van Der Mei et al., 1993). These have been described as having potential, although none of these studies were able to report that the technique in question was able to resolve all of the currently recognized species as delineated by whole genomic DNA-DNA hybridization. Monoclonal antibodies have been used for the enumeration of certain species within the mutans streptococci (De Soet and De Graaff, 1990), but this technology has not been expanded to encompass the other species groups.

Identification of oral streptococci based on genotype provides another broad approach, and several strategies have been tried, although as yet no comprehensively reliable protocols suitable for routine use have become established. Restriction fragment length polymorphism (RFLP) patterns from genomic digests were quickly found to be too complex to be practicable (Rudney et al., 1992), and although simplified patterns could be obtained by revealing a subset of bands through Southern blotting and probing with specific gene probes or ribotyping, the practicalities of using such a system for large numbers of strains on a routine basis was questionable (Rudney and Larson, 1993; Rudney and Larson, 1994). The availability of technology for carrying out polymerase chain reactions (PCRs) together with the relative ease of high throughput DNA sequencing have provided other tactical approaches to identifying oral streptococci. Broadly speaking, these methods can be separated into those relying on PCR amplification of regions of the streptococcal chromosome bordered by repetitive DNA sequences, by random amplification through arbitrary priming, and

those that are focused on amplification and/or sequencing of specific genes or gene fragments. Alam et al. (1999) examined the potential of standard repetitive extragenic palindromic (REP)-PCR, enterobacterial repetitive intergenic consensus PCR, and *Salmonella enteritidis* repetitive element-PCR to distinguish between strains of virtually all the currently recognized species of oral streptococci and found these approaches were too discriminating to allow species-level identification but were more suitable for strain typing in ecological/epidemiological studies. Other studies have tended to evaluate the applicability of PCR-based approaches to particular areas of interest within the oral streptococci rather than attempting to develop a protocol for identifying all the recognized species. In recent studies of essentially the mutans group, randomly amplified polymorphic DNA analysis (RAPD) and arbitrarily primed PCR (AP-PCR) have been used to differentiate *S. mutans* and *S. sobrinus*, the two members of the mutans species group that are associated with humans (Truong et al., 2000; Li et al., 2001), and primers specific for the amplification of the *dexA* gene have been applied to the specific detection and identification of *S. mutans* (Igarishi et al., 1996). The AP-PCR method (utilizing a sequential two-primer strategy) has been reported to allow identification of mitis group streptococci, in close agreement with groupings based on phenotypic criteria (Rudney and Larson, 1999).

Identification of oral streptococci on the basis of genetic heterogeneity revealed by PCR amplification of specific genes or gene fragments represents a resource of enormous potential. Studies carried out so far have included those focused on the ribosomal RNA operon (Whiley et al., 1995; Shiroza et al., 1998; Sultana et al., 1998), transfer DNA and intergenic spacer length polymorphisms (De Gheldre et al., 1999), D-alanine (D-ala): D-ala ligases (Garnier et al., 1997), and manganese-dependent superoxide dismutases (Poyart et al., 1998; Kawamura et al., 1999).

In summary, relatively simple strategies exploiting genetic heterogeneity to effect reliable identification of the currently recognized oral streptococcal species are still awaited. Until such approaches become established for use in diagnostic laboratories, routine identification is best achieved through the use of key biochemical test schemes either as in-house tests or incorporated in commercial test kits.

Genomics

To date, the only member of the oral streptococci (excluding *S. pneumoniae*) to have its complete genome sequenced is *S. mutans* (serotype c), strain UA159. This has been carried out by the Genome Center and the Department of Microbiology and Immunology at the University of Oklahoma in the United States. According to the latest information at the time of writing, sequencing pUC subclones from shotgun libraries and closure had yielded one contiguous sequence of 2,032,327 bp. The average G+C content is 37 mol%, and of the 1,469 open reading frames (ORFs) identified, 1,265 (>86.1%) have homologues, 787 (ca. 53%) contain superfamily assignments, and 36.1% are assigned to functional categories. The sequence data is available. A preliminary report has been published describing some of the features of approximately 90% of the genome that had been covered at that point (Ajdic et al., 2000).

Oral Streptococci and Disease

In addition to comprising a significant proportion of the normal microbial flora of the mouth in healthy individuals, oral streptococci are involved in several different types of disease (Hardie and Marsh, 1978b; Hardie and Whiley, 1994). These include conditions that occur locally in the mouth, such as dental caries and dental abscesses, as well as a variety of pathological processes in other parts of the body, including infective endocarditis and abscesses in various organs. Oral streptococci also can cause serious infections in immunocompromised patients. Pyogenic infections arising in and around the oral cavity, which frequently involve streptococci together with a number of anaerobes, may spread to other regions by the bloodstream, the lymphatics, or by direct extension along fascial planes (Schlossberg, 1987). Such infections may be extremely severe and life threatening, particularly when they cause obstruction of the airway or spread to the brain. Rarely, other conditions such as spondylodiscitis can also be caused by oral streptococci (Weber et al., 1999).

Dental Caries

Although, theoretically, any acidogenic bacteria in dental plaque may contribute to the demineralization of enamel responsible for the initiation of dental caries, most studies in recent years have been focused primarily on *Streptococcus mutans* or the mutans streptococci (MS). The extensive literature on *S. mutans* since the original description of the species by Clarke (1924) has been fully reviewed by others (Hamada and Slade, 1980; Hamada et al., 1986; Loesche, 1986), and no attempt will be made here to cite

all the many hundreds of publications on this subject.

ANIMAL STUDIES The most direct evidence for the cariogenic potential of different species of streptococci and some other genera has come from studying experimental infections in germ-free or gnotobiotic animals. Since the early studies (Orland et al., 1955; Fitzgerald and Keyes, 1960), which showed that monoinfections of rats and hamsters could induce caries and that the disease was transmissible from one animal to another (Keyes, 1960), many different bacterial strains have been tested in a variety of animal model systems (Fitzgerald, 1968; Tanzer, 1981).

When cariogenicity was first demonstrated in animals, the taxonomy and nomenclature of the streptococci tested was less well defined than it is now. It is clear that members of the MS, and *S. mutans* in particular, have the ability to induce caries in animals, but several other species have also been shown to have some cariogenic potential, including certain strains of the anginosus group (formerly referred to as "*S. milleri*" (Drucker and Green, 1978), *S. salivarius* (Drucker et al., 1984b) and *S. oralis* (Willcox et al., 1987). In contrast, strains of *S. sanguis* and *S. mitis* are reported to produce little or no caries in gnotobiotic rats (Drucker and Green, 1978; Drucker et al., 1984a; Fitzgerald, 1968).

Although some differences are evident, owing to variations in the types of animals used, bacterial strains tested, and experimental conditions employed, it appears that most strains of *S. mutans* are able to produce high levels of caries, usually affecting different types of tooth surface (e.g., fissures and smooth surfaces). With other species, not all strains are cariogenic, the overall level of caries is generally less than that produced by *S. mutans*, and the lesions may be restricted to particular tooth surfaces. Thus, there is a spectrum of caries-inducing potential among the oral streptococci, with *S. mutans* being most active and other species, such as *S. sanguis* and *S. mitis* displaying negligible cariogenic activity.

HUMAN STUDIES Many studies have been undertaken to establish the relationship between specific oral bacteria and dental caries in various human populations, most of which have concentrated on MS and lactobacilli as the putative cariogenic organisms (Loesche, 1986). In addition to examining different groups of subjects of different ages, country of origin, disease status, and other variables, such studies have varied by being either cross-sectional or longitudinal in design. Since dental caries develops in a person over a period of time and is difficult to diagnose in the initial stages, especially in the depths of occlusal fissures and on proximate surfaces between the teeth, the longitudinal approach is preferable when attempting to demonstrate cause and effect relationships.

Notwithstanding problems associated with experimental design and techniques, many studies have reported a strong association between MS, particularly *S. mutans*, and human caries, using either dental plaque or saliva as samples (see Krasse, 1988; Loesche, 1986; MacFarlane, 1989; Rudney and Larson, 1993, and Hardie and Whiley, 1999 for reviews). A similar, albeit less strong, association has also been recorded between lactobacilli and human caries, but it is not entirely clear whether these bacteria are involved in initiation or subsequent progression of the lesions. Although the weight of the published evidence supports the concept of a key role for *S. mutans* in most types of human caries (e.g., Loesche et al., 1984; Kristoffersson et al., 1985), some longitudinal studies have failed to demonstrate a clear association between this species and the initiation of caries at specific sites (Mikkelsen and Poulsen, 1976; Hardie et al., 1977; Mikkelsen et al., 1981), thus giving some credence to the possibility that occasionally other organisms may be responsible. This would be in accord with the evidence from animal experiments and with the suggestion that the flora associated with dental caries may vary at different stages of disease progression (Marsh et al., 1989).

VIRULENCE FACTORS The cariogenicity of the mutans streptococci is due to their ability to colonize the tooth surface, forming a constituent of dental plaque, and to produce acid. It has long been known that dietary sucrose plays an important etiological role in dental caries, and a considerable amount of research has been carried out on the metabolism of sucrose by the mutans streptococci (Hamada and Slade, 1980; Loesche, 1986). In addition to producing acid from sucrose, *S. mutans* and *S. sobrinus* also utilize this substrate for the formation of extracellular glucans and fructans. Various structural forms of these polymers are produced which may be cell-associated or released into the surrounding environment, and their formation depends upon the activities of different glucosyl- or fructosyl-transferases. Depending on the specific linkages present, these extracellular polysaccharides may be water soluble or insoluble. One particular water-insoluble α-(1-3)-glucan of *S. sobrinus* (originally referred to as "*S. mutans*") is known as mutan (Guggenheim, 1970). In addition to extracellular polysaccharides, *S. mutans* strains produce an amylopectin-like intracellular polysaccharide, which can be metabolized when external sources of carbohydrate are unavailable.

Mutant strains of *S. sobrinus* and *S. mutans* with reduced ability to produce extracellular polysaccharides have been shown to have lower caries-inducing potential in experimental animals (De Stoppelaar et al., 1971; Tanzer et al., 1974; Michalek et al., 1975; Johnson et al. 1977; Otake et al., 1978; Freedman et al., 1981; Freedman et al., 1983). Similarly, mutants of *S. mutans* with decreased acid production (Mao and Rosen, 1980), decreased aciduricity (De Stoppelaar et al., 1971), or decreased intracellular polysaccharide production (Tanzer et al., 1976) are also less cariogenic in animals. The absence of lactate dehydrogenase (LDH) in mutants of *S. rattus* is also associated with markedly reduced caries-inducing ability (Johnson et al., 1980). More recently, modern genetic analysis and cloning techniques have been applied to the study of virulence determinants of the mutans streptococci (Curtiss et al., 1986b; Russell, 1994)

Caries Vaccine   Since the 1970s, work has been in progress in a number of laboratories to develop an anti-caries vaccine based on *S. mutans*. A variety of preparations have been tested in rodents and primates, including vaccines initially based on whole cells, crude cell wall preparations, glucosyltransferases, and several purified protein antigens (for reviews of earlier studies, see Lehner et al., 1981; McGhee and Michalek, 1981; Cohen et al., 1983; Curtiss, 1986a; Hamada et al., 1986; Russell and Mestecky, 1986; Russell and Johnson, 1987b, and Klein and Scholler, 1988). Reservations have been expressed about the desirability of such vaccines, partly because of concern about safety and the possibility of inducing antibodies against heart tissue (antibodies with which *S. mutans* may crossreact), and partly because of the decrease in caries in many industrialized countries and the availability of other effective and totally safe caries-preventive measures. However, since dental caries is still prevalent in many parts of the world, a vaccine could be an extremely valuable adjunct to other preventive approaches.

The exact mechanisms by which *S. mutans* vaccines exert their caries-preventive effect in animals are not fully understood, and both systemic immunoglobulin (Ig)G and local IgA antibody responses have been considered to be significant in protection. However, in recent times, more emphasis has been put on the development of mucosal vaccines that stimulate secretory immunity (Russell et al., 1999; Hajishengallis and Michalek, 1999; Smith et al., 2001). The application of molecular biological techniques has allowed the development of more specifically defined antigen preparations, including surface adhesins and glucosyltransferases. In one recent study, intranasal immunization of mice with a vaccine prepared from the glucan-binding region of *S. mutans* glucosyltransferase produced a significant reduction in colonization and protected against the development of caries (Jesperaard et al., 1999). Stimulation of the local secretory immune response in infants might prevent the colonization of newly erupted teeth by mutans streptococci and be an effective approach to caries prevention (Michalek et al., 2001).

A different approach to immunization against dental caries is to use topical application of preformed antibodies to mutans streptococci. Recent work on passive immunization with antibodies produced by genetically engineered green plants shows considerable promise and the results of ongoing clinical trials are awaited with interest (Ma, 1999).

Purulent Infections

Purulent infections in various parts of the body are frequently found to be associated with mixed bacterial flora, often involving streptococci together with two or more obligate anaerobes. This is particularly evident with dento-alveolar abscesses where up to eight isolates per specimen may be recovered, the mean number usually being between three and five per abscess (Heimdahl et al., 1985; Lewis et al., 1986; Sakamoto et al., 1998).

Many workers have noted the association of the *S. anginosus* ("*S. milleri*") group with purulent infections since their occurrence in dental abscesses was first described by Guthof (1956). The extensive literature on the clinical significance, occurrence, and pathogenicity of these streptococci has been reviewed by several authors (Van der Auwera, 1985; Gossling, 1988; Ruoff, 1988), and case reports of particular infections have continued to appear regularly. The list of sites and conditions from which anginosus group streptococci have been reported is impressive and includes dental abscess, maxillary sinusitis, brain abscess, meningitis, pharyngitis, lung abscess, pleural empyema, liver abscess, intra-abdominal abscess, peritonitis, appendicitis, female genital tract, neonatal sepsis, spinal epidural abscess, bone and joint infections, pacemaker infection, vascular graft infection, and assorted other infections.

The confusion that has surrounded the taxonomy and nomenclature of the anginosus group, as described above, means that some of the earlier epidemiological and ecological data are difficult to interpret, since the precise identity of the streptococci isolated is not always clear. For example, where the isolates have been described simply as *S. milleri*, it would be preferable to determine which of the currently recognized

species (i.e., *S. anginosus*, *S. constellatus* or *S. intermedius*) they resemble. Unfortunately, even when these specific epithets have been used, the identifications may not always be reliable if based solely on conventional biochemical and physiological characters of the strains. In other cases, different designations such as "group F streptococci" have been used to describe the isolates found (Libertin et al., 1985; Shlaes et al., 1981).

Studies on almost 300 well-characterized isolates from a variety of clinical infections at different body sites have shown striking differences in the distribution of *S. anginosus*, *S. constellatus* and *S. intermedius* (Table 7).

The apparently strong association of *S. intermedius* with the central nervous system, especially brain abscesses, and the frequency of isolation of *S. anginosus* from genitourinary and gastrointestinal specimens are of particular interest (Whiley et al., 1990b; Whiley et al., 1992; Hardie and Whiley, 1994). A survey of oral samples from healthy individuals showed *S. anginosus* is the most commonly isolated species (Whiley et al., 1993).

Infections with the anginosus group are generally thought to have an endogenous origin, inasmuch as these organisms form part of the resident flora of the mouth and upper respiratory tract, the gastrointestinal tract, and the female urogenital tract. However, the exact site of origin of the infecting organism may not always be obvious, especially when the pathogen has reached the affected organ via the bloodstream. Some patients with debilitating conditions such as malignancies, blood dyscrasias, reduced immunity due to immunosuppressive therapy or diabetes mellitus, develop purulent infections with these streptococci (as well as other organisms), although such predisposing conditions are not essential for infections with the anginosus group.

It has been reported that prophylaxis with antimicrobial agents such as metronidazole and aminoglycoside antibiotics prior to abdominal surgery can predispose to suppurative, postoperative infections with the anginosus group (Tresadern et al., 1983), and this may occur following appendectomy in children (Madden and Hart, 1985). Such streptococcal infections may be facilitated by the suppression of other components of the gut flora, especially the anaerobes, by these antimicrobial agents.

Pathogenicity of the Anginosus Group

Experimental infections in animals with (either pure or mixed cultures of) anginosus group streptococci have been reported by several investigators (see Gossling, 1988 for review). Relatively large inocula (10^8–10^{10} cells) have generally been required to produce suppurative infections (Brook and Walker, 1984). Experimental infections in mice with bacteria from dental abscesses indicated that anginosus group strains were less virulent than the anaerobic Gram-negative species tested, although they nevertheless did induce abscess formation (Lewis et al., 1988).

Several possible virulence factors have been described, including hyaluronidase (Kilpper-Bälz et al., 1984), gelatinase and collagenase (Steffen and Hentges, 1981), and DNase and RNase (Pullian et al., 1980; Marshall and Kaufman, 1981). One taxonomic study indicated that hyaluronidase activity may be a constant characteristic of strains in the same DNA similarity groups as *S. intermedius* and *S. constellatus*, but this enzyme was not found in any of the 12 strains of *S. anginosus* examined (Whiley and Hardie, 1989). An immunosuppressive fraction from a strain of *S. intermedius* that strongly suppresses lymphocyte and fibroblast proliferation has also been described (Arala-Chaves et al., 1981).

One potential virulence factor shown to be of significance in experimental infections with these streptococci is the possession of a polysaccharide

Table 7. Numbers of clinical isolates of *S. anginosus*, *S. constellatus* and *S. intermedius* associated with different anatomical sites.

Site of infection	Number of strains			Total
	S. anginosus	*S. constellatus*	*S. intermedius*	
Central nervous system	2	2	23	27
Head and neck	21	35	42	98
Respiratory tract	7	9	1	17
Gastrointestinal tract	26	8	2	36
Abdominal and pelvic	6	7	11	24
Genitourinary tract	51	6	0	57
Skin, soft tissue and bone	15	6	7	28
Blood	5	5	1	11
Total	133	78	87	298

Data from Whiley et al., 1990b, 1992; and Hardie and Whiley, 1994.

capsule. Pure cultures of strains with >50% of cells encapsulated (unlike a nonencapsulated strain of *S. intermedius*) were found to be capable of inducing abscesses (Brook and Walker, 1985).

Surface-associated properties and the ability to bind to albumin and other host molecules may also be relevant to the pathogenic potential of these streptococci (Willcox et al., 1993).

A novel cytotoxin, specific for human cells, has been identified and purified from a strain of *S. intermedius* originally isolated from a human liver abscess (Nagamune et al., 1996). This cytotoxin, named "intermedilysin" (ILY), is a 54-kDa protein with between 42–71% primary sequence homology to the thiol-activated pneumolysin of *S. pneumoniae*, based on amino acid sequence homology to five internal sequences of ILY. It is strongly cytotoxic to human cells, but not to those of other animal species tested.

Distribution of the ILY gene amongst anginosus group streptococci, as determined by PCR and Southern hybridization, appears to be confined to strains of *S. intermedius*. No homologue to the toxin gene was detected in strains of *S. anginosus* or *S. constellatus* (Nagamune et al., 2000). Also, ILY production was found to be higher from clinical isolates of *S. intermedius* from deep-seated infections when compared to oral (normal flora) isolates, suggesting that this cytotoxin may be a significant factor in such infections. Purified ILY (40 ng/μl) causes a significant reduction in intact polymorphonuclear cells (PMNs) after 60 minutes in vitro (Macey et al., 2001). Active ILY was not found to be chemotactic when compared to heat-inactivated cytolysin, but did cause an increase in other indicators of PMN activity, which may be of some relevance in deep-seated infections (Macey et al., 2001).

Infective Endocarditis

The oral (viridans) streptococci have long been recognized as important etiological agents in endocarditis. Bacteremias arising from the mouth, as during tooth extraction and other dental operative procedures, invariably involve oral streptococci, and these may settle on the endocardium in "at risk" patients with preexisting heart valve lesions (Hardie and Marsh, 1978b). However, it should be noted that the oral cavity is not the only source of such transient bacteremias, and in a significant proportion of cases of endocarditis, the organisms may enter the bloodstream from the gut, genitourinary tract, or other body sites (Bayliss et al., 1983).

Several investigators have reported a decrease in the proportion of cases of infective endocarditis caused by viridans streptococci during the latter part of the twentieth century (Roberts et al.,

1979; Bouvet and Acar, 1984). Streptococci continued to be the most commonly isolated organisms in most studies, but the relative numbers of enterococci, staphylococci and a variety of other organisms appeared to increase, particularly in older patients. Notwithstanding this apparent shift in the pattern of bacteria associated with the disease, one recent study of 128 episodes of infective endocarditis in Wales still showed viridans streptococci to be the predominant group of organisms isolated (Dyson et al., 1999).

Several groups have identified the streptococcal species isolated from patients with infective endocarditis (e.g., Parker and Ball, 1976; Facklam, 1977; Lowes et al., 1980; Moulsdale et al., 1980; Bayliss et al., 1983; Bouvet and Acar, 1984; Horaud and Delbos, 1984). Direct comparisons between such earlier studies are complicated by variations in the taxonomic systems employed by different authors. *Streptococcus sanguis* and *S. oralis* (the latter often reported as *S. mitis*, "*S. mitior*" or *S. sanguis* II) have been isolated most often in these series, but significant numbers of *S. mutans* and the anginosus group streptococci have also been recorded. At least some isolates identified in the past as *S. sanguis* may have been representative of *S. gordonii* (Kilian et al., 1989b).

In one more recent study of 47 strains of streptococci from 42 cases of infective endocarditis, using current nomenclature, the most commonly identified species were *S. sanguis* (32%), *S. oralis* (30%) and *S. gordonii* (13%). Smaller numbers of *S. bovis* (6%), *S. parasanguis* (4%), *S. salivarius* (4%) and *S. mutans* (4%) were also recorded in this series (Douglas et al., 1993).

The importance of distinguishing between *S. bovis* (biotypes I and II) and *S. salivarius*, when identifying strains from bacteremias, has been pointed out by Ruoff et al. (1989). These authors noted a striking association between *S. bovis* I bacteremia and underlying endocarditis, and also confirmed the previously reported correlation between this organism and the presence of colonic neoplasms (Klein et al., 1979).

The exact prevalence of each of the oral streptococci is difficult to determine from published reports, since some studies are based on a series of cases of endocarditis at a particular center over a given period, whereas others depend on analyses of isolates sent to a reference laboratory for identification.

A number of possible virulence determinants have been described in endocarditis-inducing streptococci. These include production of extracellular polysaccharides, ability to aggregate human platelets, and attachment to fibronectin, laminin and other cellular components (Herzberg et al., 1992; Sommer et al., 1992; Manning et al., 1994).

The diagnosis of infective endocarditis is normally confirmed in the laboratory by isolation of the causative organism from blood cultures, but in up to 10% of cases, such cultures may be negative (Bayliss et al., 1983).

Infections in Immunocompromised Patients

Opportunistic infections with a wide range of microorganisms are common in patients who are immunocompromised or debilitated in some way, due to a variety of underlying causes. In many cases, such infections are associated with bacteria that are not normally regarded as highly virulent or invasive pathogens. In neutropenic patients (those with a decreased number of neutrophils in the blood), there has been a shift in the pattern of infection over the last 20 years or so with a dramatic increase in the number of reported cases due to Gram-positive bacteria (including enterococci and viridans streptococci; Oppenheim, 1998). Oral streptococci are now recognized as an important cause of serious, life-threatening infections in neutropenic patients, leading to conditions such as septicemia and the adult respiratory distress syndrome (ARDS).

From several studies where the causative streptococci in such cases have been identified to species level, it appears that streptococci from the mitis group, especially *S. oralis*, *S. mitis* and *S. sanguis*, are a significant cause of life-threatening infections in immunocompromised individuals (Beighton et al., 1994).

Neutropenic patients with streptococcal infection sometimes develop toxic shock syndrome. Viridans streptococci from such cases have been shown to induce tissue necrosis factor alpha (TNFα) from murine macrophages (Orlicek et al., 1997), and TNFα, TNFβ and interleukin 8 (IL-8) from human blood mononuclear cells in vitro (Soto et al., 1998). The increased induction of IL-8 by clinical isolates compared to commensal strains may be of particular significance in ARDS.

Another property of some of the streptococci found in these infections is the production of sialidase (exo-glucosidase). Both *S. oralis* and *S. intermedius* produce this enzyme, which is thought to be a key factor for growth of these organisms on glycoproteins in vivo and may also be relevant to their ability to produce infections at extra-oral sites of the body (Byers et al., 1999; Byers et al., 2000).

A number of species of oral streptococci have been isolated from cervical lymph nodes in patients with oral cancer. These include *S. intermedius*, *S. constellatus*, *S. oralis*, *S. mitis*, *S. sanguis* and *S. salivarius* (Sakamoto et al., 1999). Streptococci were the most common isolates from such lymph nodes, although several enteric bacteria and *Peptostreptococcus* species were also recovered.

Meningitis

Oral streptococci, other than *S. pneumoniae*, are occasionally isolated from blood or cerebrospinal fluid of patients with bacterial meningitis. In cases where anginosus group streptococci are recovered, this may be an indication of an undiagnosed brain abscess, whereas the isolation of other oral streptococci may be associated with concurrent infective endocarditis (Cabellos et al., 1999). Oral streptococci represented 5% of culture positive cases in one prospective study of meningitis in adults (Enting et al., 1997)

Antibiotic Susceptibility of Oral Streptococci

The species of streptococci considered in this chapter have generally been thought to be fully susceptible to penicillin, although the existence of resistant strains has been known for many years (Garrod and Waterworth, 1962; Naiman and Barrow, 1963; Phillips et al., 1976). Examination of one series of strains from infective endocarditis, excluding enterococci, showed that 91% of strains were sensitive to benzylpenicillin (Etienne et al., 1984). The penicillin-resistant strains were identified in this study as *S. sanguis* I, *S. sanguis* II and *S. mitis*. Resistance to erythromycin was found in 16% of strains. In another study of endocarditis isolates (Horaud and Delbos, 1984), 96% of strains were sensitive to penicillin, but 20% were resistant to tetracycline, and 8% were found to be multiply resistant.

Serious, life-threatening infections due to penicillin-resistant oral streptococci (including *S. intermedius* and *S. mitis*) have been reported, and penicillin resistance may be associated with altered penicillin-binding proteins (Quinn et al., 1988). Tolerance to penicillin has also been observed amongst oral streptococci, in which the lethal (bactericidal) effect of the antibiotic is greatly reduced (Slater and Greenwood, 1983; Handwerger and Tomasz, 1985; Catto et al., 1987; Powley et al., 1989). Thus, as pointed out by Quinn et al. (1988), it is desirable to report antibiotic-sensitivity results on all isolates of oral streptococci from sites that are normally sterile.

Children who receive repeated courses of antibiotic treatment for conditions such as otitis media and cystic fibrosis are more likely to become colonized by resistant strains of oral streptococci than healthy control subjects (Alvarez et al., 1998; Erickson and Herzberg, 1999).

Since it is known that penicillin-resistant oral streptococci act as the genetic reservoir for β-lactam resistance in *S. pneumoniae*, the high levels of resistance observed in some oral species, such as *S. mitis* and *S. oralis*, may be of considerable clinical significance (Konig et al., 1998; Teng et al., 1998). The reported differences in susceptibility between streptococcal species underline the importance of accurate identification of clinical isolates and the need for continuing surveillance of antimicrobial resistance.

Molecular Studies on Oral Streptococci

As reviewed in other sections, the application of molecular and genetic techniques, such as nucleic acid hybridization and 16S rRNA sequencing, has had a considerable impact on our current understanding of the taxonomy of the oral streptococci (Schleifer and Kilpper-Bälz, 1987). The development of DNA probes for identification of streptococci, as described for *S. oralis* (Schmidhuber et al., 1988), is likely to be extended to cover other species. For epidemiological studies, the technique of DNA fingerprinting by restriction pattern analysis provides another potentially useful tool (Skjold et al., 1987), and this has already been applied in a study on transmission of *S. mutans* (Caufield and Walker, 1989).

Streptococcus mutans has probably received more attention than any other oral microorganism, with many studies on various aspects of the molecular biology of sucrose metabolism, surface antigens, plasmids, and the development of vaccines (Hamada and Slade, 1980; Hamada et al., 1986; Loesche, 1986). Genetic approaches to the study of virulence have been extensively used in research on *S. mutans* (Curtiss, 1986a; Russell and Gilpin, 1987a), and other oral streptococci are beginning to receive similarly detailed examination (Russell, 1990; Jacques, 1998). Such studies should lead to a greatly enhanced understanding of the mechanisms by which oral streptococci colonize the mouth and cause disease. With the anticipated further development of specific probes against these streptococci, it will also become possible to undertake far more sophisticated studies on their ecology, epidemiology, and role in various disease processes.

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CHAPTER 1.2.3

Medically Important Beta-Hemolytic Streptococci

PATRICK CLEARY AND QI CHENG

Introduction

Streptococci are a diverse collection of species, which are Gram-positive cocci that grow in chains or pairs. Although most are normal flora of mammalian mucous membranes, all can cause disease, and some are primary pathogens. With the exception of *Streptococcus pneumoniae*, the most medically relevant species are β hemolytic when grown on sheep blood agar plates. *Streptococcus pyogenes* and *S. agalactiae* are by far the most common, most dangerous and best studied human streptococcal pathogens, and are therefore the primary focus of this chapter.

Streptococci are fastidious chemoorganotrophs, which are catalase negative. All grow aerobically, but growth is more luxurious in an atmosphere that contains elevated carbon dioxide (CO_2) levels. Although unable to utilize oxygen (O_2) as an electron sink, it can be metabolized to reactive oxygen radicals and hydrogen peroxide (H_2O_2). Strains within a species vary in their sensitivity to O_2 and excretion of H_2O_2. *Streptococcus pyogenes* and others produce manganese (Mn)-dependent superoxide dismutases (Gerlach et al., 1998). Streptococci are unable to synthesize hem, the basis for their negative response to the benzidine and the porphyrin tests (Ruoff, 1991).

A hallmark of most pathogenic species of streptococci is β hemolysis. Five percent washed sheep red blood cells in agar medium are most often used to evaluate hemolysis. Both the diameter and transparency of the zone of hemolysis can vary from species to species, within a species, even among colonies of a single culture (Fig. 1). Panels A and B (Fig. 1) show the morphology and hemolysis of a typical *S. pyogenes* culture. Agar plates are often stabbed in the first streak quadrant to reveal more intense hemolysis. Both the zone of hemolysis and colony size varies from culture to culture. Panel C shows an *S. pyogenes* culture that primarily makes α hemolytic colonies but occasionally segregates a brightly β hemolytic colony that is more typical. *Streptococcus agalactiae* usually forms smaller colonies with a smaller zone of β hemolysis (Fig. 1, Panel

D). Human group C and G clinical isolates are also β hemolytic (Fig. 1, Panels E and F, respectively). The group G culture shown in Panel F (Fig. 1) also demonstrates variable β hemolysis. Therefore, β hemolysis is not a particularly useful characteristic for speciation or subspeciation of cultures. Neither the source of this variability nor the genetic regulation of hemolysins has been studied. Because pathogenic species are usually β hemolytic, it has been assumed that hemolysins contribute to the pathogenesis of streptococci. Convincing evidence for this assumption is still, however, lacking. Most hemolysins are poorly defined. The one exception is streptolysin O, and it will be discussed in more depth in another section (Adderson et al., 1998).

Genetic variability is an important, often overlooked characteristic of pathogenic streptococci in general. Changes in colony morphology, growth characteristics of broth culture and expression of both surface and extracellular proteins are common. Phase-variation in colony morphology with concomitant loss or gain in virulence has been described for both *S. pyogenes* (Simpson and Cleary, 1987a) and *S. agalactiae* (Pincus et al., 1993; Akesson et al., 1996). The genetic switch that is responsible for phase variation has not been defined, nor can it be controlled in the laboratory. It is likely laboratory cultures that have been passed many times deviate considerably from the original specimens isolated from patients. This is a source of concern to researchers who produce mutants as a means to study the impact of a given gene on virulence. The rate of spontaneous change is often greater than that of induced mutations; therefore, it is essential to backcross putative mutations into wild type cultures, or isolate revertants to confirm that the change in phenotype was produced by the mutation (Alouf and Loridan, 1988).

Methods for isolation and identification of β hemolytic streptococci will not be described here. Biochemical and serological procedures have changed little from the last edition of this book. Readers are also referred to Johnson et al. (1996), also published in other languages, for

Fig. 1. Comparison of colony morphologies of β-hemolytic streptococci. Panels A, B and C are *S. pyogenes* colonies. Panels D, E and F are *S. agalactiae*, group C and G streptococci, respectively.
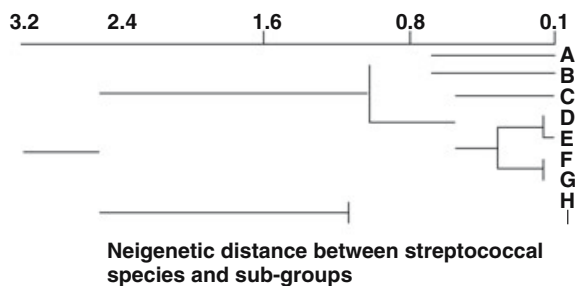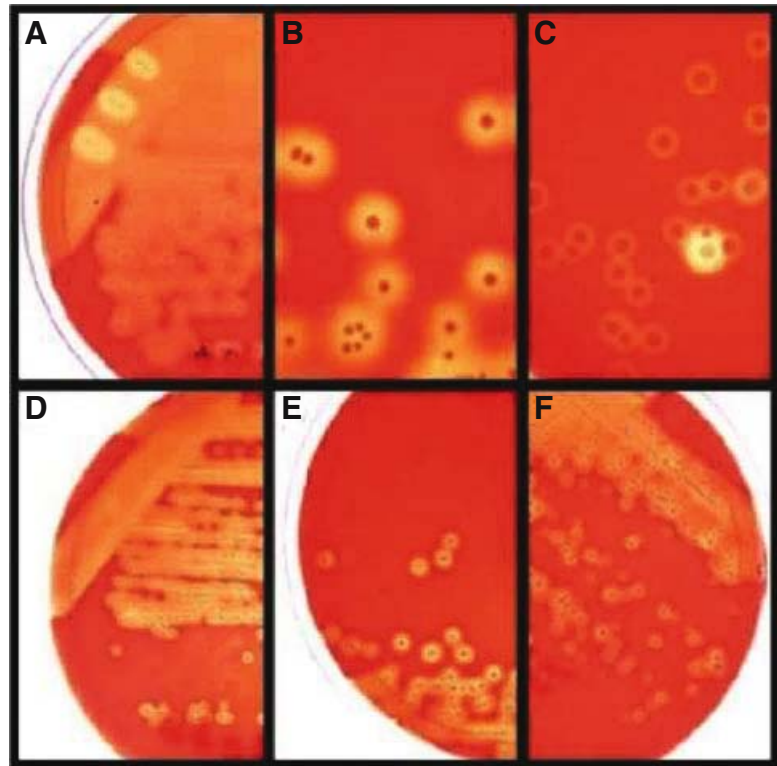
Fig. 2. Phylogenetic tree of streptococcal species. Letter indicates the species, Lancefield group, or subspecies. A = *S. pyogenes*, B = *S. canis*, C = human group C, G = human group G, L = human group L, C′ = animal group C, H = *S. equi*, I = *S. iniae*. Genetic distance was determined by Vieira et al. (1998). ["L and C′" mentioned in the figure legend is not seen in the schematic.]

identification and serological characterization of *S. pyogenes*. Good sources of information for streptococci in general are also available (Facklam et al., 1979; Facklam and Carey, 1985).

Although species boundaries have become clearer with the application of modern taxonomic tools, differences often fade when characteristics required for colonization of mammalian hosts are considered. Different species of human origin often express common determinants of virulence. For example, *S. pyogenes* and *S. agalactiae* express complement (C5a)-specific proteases (i.e., the C5a peptidase) that are greater than 95% identical in amino acid sequence (Chmouryguina et al., 1996). Group C and G streptococci express M proteins that are highly similar to those produced by *S. pyogenes* (Anthony et al., 1981). The fact that cultures from nonhuman sources fail to express these proteins suggests that the genes encoding these virulence factors were acquired subsequent to the evolution of species boundaries. Virulence genes have been transmitted horizontally between species, but this has not been demonstrated in the laboratory. As observed among enteric pathogens, the genome of this genus is remarkably plastic. Plasmids are more rare in streptococci than enteric bacteria. Conjugative transposons and temperate bacteriophage are likely to be more important vectors of horizontal gene transfer.

Taxonomy

Medically important β-hemolytic streptococci were originally speciated into groups, based upon the serological specificity of their cell wall C carbohydrates. Groups A–V and provisional groups W–Z, with the exception of I and J, are now recognized (Facklam and Edwards, 1979). Teichoic acids account for the group antigens of groups D, N, and Q. Group antigens can be extracted by several methods and assayed by precipitation, agglutination, immunofluores-

Table 1. Nomenclature of β-hemolytic streptococci.

Species	Colony size	Lancefield group antigen	Remarks
S. pyogenes [35]	Large	A	Some isolates of *S. anginosus* produce the A antigen, but this species forms smaller colonies
S. agalactiae [36]	Large	B	Human and bovine strains are closely related but can be distinguished biochemically
S. dysgalactiae [15]			
subsp. *equisimilis*	Large	C, G, or L	Associated with human disease and express many of the same virulence factors that are produced by *S. pyogenes*
subsp. *dysgalactiae*	Small	C	Either α or nonhemolytic, infections of veterinary importance
S. equi			
subsp. *equi*	Large	C	Strangles horses
subsp. *zooepidemicus*			Normal flora in horses and occasional cause of human diseases
S. canis	Large	G	Dog and cat infections
S. anginosus	Small	A, C, G, or F	Belong to the "streptococcus milleri group"

Table 2. Biochemical tests used for differentiation of streptococci.

	Susceptibility to:		PYR	CAMP test	Hydrolysis of hippurate	Bile esculin	Growth in 6.5% NaCl	Optochin and bile susceptibility
	Bacitracin	SXT						
S. pyogenes (group A)	S	R	+	−	−	−	−	R
S. agalactiae (group B)	R[a]	R	−	+	+	−	+[a]	R
S. dysgalactiae (group C and G)	R[a]	S	−	−	−	−	−	R
S. pneumoniae		S	−	−	−	−	−	S
S. bovis	R		−	−	−	+	−	R

Symbols: S, sensitive or susceptible; R, resistant; +, positive; −, negative; SXT, sulfamethoxazole and trimethoprim; PYR, pyrrolidonyl arylamidase; CAMP test, test for enhancement of hemolysins by *Staphylococcus aureus* beta lysin.
[a]Exceptions occasionally.
Adapted from Ruoff (1991) and other sources.

cence or ELISA assays using appropriate antisera. Although group antigens are used to speciate streptococci, this approach has produced confounding taxonomic relationships. Clinicians and researchers in the field, however, still primarily use these markers and strain designations. Table 1 (Bohnsack et al., 1997) lists the most important β-hemolytic species and a few general characteristics of each. The species boundaries of *S. pyogenes* and *S. agalactiae* are well defined. The others can be placed in distinct ecovars, even though they may share the same group antigen. The pathogenesis literature primarily uses the Lancefield group to designate species, and that trend will be continued for the various *S. dysgalactiae* subspecies.

Biochemical testing and genetic analyses argue for medically important β-hemolytic streptococci to be divided into three species: *S. pyogenes*, *S. agalactiae* and *S. dysgalactiae* (Table 2). Two other species that are sometimes β hemolytic and that occasionally cause human disease are *S. anginosus* and *S. suis*. The β-hemolytic subspecies of *anginosus* produce

hyaluronidase and group C carbohydrate antigen (Turner, 1997).

Taxon *Streptococcus pyogenes*

IDENTIFICATION Although rapid tests for *S. pyogenes* are commercially available, throat cultures on sheep blood agar plates remain the gold standard (Kaplan, 1997). Johnson et al. (1996) published an excellent manual that describes methods used for laboratory identification of *S. pyogenes*. Therefore, this section will concentrate on markers used to subspeciate clinical isolates for epidemiological studies. *Streptococcus pyogenes* is divided into two subspecies (OF[+] and OF[−] serotypes) based on the production of serum opacity factor, a lipoproteinase that turns horse serum cloudy (Saravani and Martin, 1990). More recently, OF[+] and OF[−] serotypes were designated class I and class II, based on presence of an antigenic epitope located in the constant domain of M protein (Bessen et al., 1989). This division of *S. pyogenes* serotypes also reflects their propensity to cause different types of infections. Class I

strains are more often associated with pharyngitis and rheumatic fever, whereas class II strains are more often responsible for impetigo (Bessen et al., 1989). The relationship between disease and serotype is not firm and is questioned by many in the field. Horizontal spread of genes encoding virulence factors (Simpson et al., 1987b; Whatmore and Kehoe, 1994) and phenotypic variability within a serotype subclass suggest a more complex situation.

Since the early work of Rebecca Lancefield, *S. pyogenes* has been subclassified for epidemiological purposes by their M (Anthony et al., 1981) and T surface antigens. Several reference laboratories around the world including at the Centers for Disease Control and Prevention (CDC) and in Minnesota, Colindale, Czech Republic, Russia, Italy and New Zealand (Table 3) produce and retain rabbit antiserum directed against these antigens. The gene that encodes a T antigen was cloned and sequenced (Jones et al., 1991). Hybridization experiments using this gene as a probe suggest that T antigens are a collection of unrelated, relatively trypsin-resistant, cell-wall-associated proteins. Agglutination of trypsin-digested, live streptococci with T typing sera yields a pattern of T agglutination that is predictive of the M serotypes (Johnson et al., 1996). Occasionally, the T type is used as a stand-alone marker of *S. pyogenes* strains. More often, the M protein is the serological label chosen to subclassify *S. pyogenes*. Production of rabbit sera against the M proteins of many OF$^+$ strains of streptococci is technically difficult. For this reason, reference laboratories have resorted to using human or guinea pig hyperimmune serum that will neutralize the serum opacity reaction (Johnson et al., 1996). The antigenic specificities

Table 3. List of Streptococcal reference laboratories and their addresses.

Name	Address	Phone	Fax	E-mail
Dr. Androulla Efstratiou	Streptococcus and Diphtheria Reference Unit Respiratory and Systemic Infection Laboratory PHLS Central Public Health Laboratory 61 Colindale Ave. London NW9 5HT, UK	(44)181 200 4400 Ext 4270	(44)181 205 6528	aefstrat@phls.co.uk
Dr. Richard Facklam, Chief	Childhood and Respiratory Diseases Branch Division of Bacterial and Mycotic Diseases NCID, CDC Mailstop, Co2 Bldg 5, Rm 23 1600 Clifton Rd., NE Atlanta, GA 30333 USA	(1)404 639 1379	(1)404 639 3123	rrf2@cdc.gov
Dr. Paul Kriz, Head of WHO Collaborating Center for Reference and Research on Streptococci	NRL for Meningococcal Infections National Institute of Public Health Srobarova 48 10042 Prague 10 Czech Republic	(420)2 6708 2259	(420)2 6731 1454	krizova@szu.cz
Dr. Diana Martin Managing Scientist, Special Pathogens	Institute of Environmental Science and Research Ltd. Communicable Disease Centre Kenepuru Dr. PO Box 50-348 Porirua, New Zealand	(6)44 237 0149	(6)44 237 2370	diana.martin@esr.crl.nz
Professor Artem Totolian	Institute of Exp. Medicine Department of Molecular Microbiology Russian Academy of Science 197376 St. Petersburg Russia	(7)812 234 2661	(7)812 234 9477	lab@suvorov.spb.su
Mrs. Marguerite Lovgren Technical Supervisor	Room 1B3.26 WMC National Centre for Streptococcus Provincial Laboratory of Public Health University of Alberta Hospitals 8440 = 112 Street Edmonton, Alberta Canada T6G 2J2	(1)780 407 9877	(1)780 407 8984	ml@bugs.uah.ualberta.ca
Edward L. Kaplan, M.D.	University of Minnesota Department of Pediatrics 420 Delaware St. SE, Rm. 820 Mayo, Box 296 Minneapolis, MN 55455	(1)612 624 1112	(1)612 624 8927	kapla001@maroon.tc.umn.edu
Dr. Graziella Orefici	WHO Collaborating Center on Streptococci and Streptococcal Diseases Lab. Batteriologia e Micologia Medica Istituto Superiore di Sanita Viale Regina Elena 299 Roma 00161 Italy	(39)6 4990 2338 or (39)6 4990 2886	(39)6 4938 7112 or (39)6 4990 2934	marella@net.iss.it (put "Graziella Orefici" in subject line since this is a general address)

of OF enzymes are distinct, but parallel those of M proteins.

Polymerase chain reaction (PCR) primers that amplify all M protein genes (Podbielski et al., 1994b) were designed and the sequencing of M protein genes (*emm*) has been standardized and automated. More than 148 distinct *emm* genes have been identified. Sequencing data revealed that a few of the original serotypes produce the same M protein, indicating that for these strains, serotype specificity is based on an antigen, other than M protein. Difficulties inherent in production and maintenance of specific sera and precision of sequence data have prompted the CDC to recommend *emm* sequencing as a means to subspeciate *S. pyogenes* (Beall, et al., 1998; Anthony et al., 1978).

DISEASE AND EPIDEMIOLOGY *Streptococcus pyogenes* was a major cause of death before and during the 19th century. Few families were left untouched by the scourge of this organism. Scarlet fever, rheumatic fever, erysipelas, glomerulonephritis and sepsis were common complications inflicted on human populations. With the industrial revolution, hygiene and living standards improved, and death from streptococcal infections dramatically declined.

Pharyngitis Strep throat is the most common infection in temperate climates (Kaplan, 1996). During late fall and winter, 30% of all visits to a general practitioner's office are associated with streptococcal pharyngitis. The incidence of strep throat is highest in school-aged children. Children under five years old, or older than 15 years, are less likely to contract this infection (Bisno, 1997b). There are exceptional adults, who experience frequent infection, and outbreaks of pharyngitis are more common in institutionalized adults, such as military personnel and individuals living in nursing homes. The organism is spread primarily by droplet infection and direct contact with saliva or nasal secretion from an infected individual. The high incidence of infection in children is thought to reflect their lack of immunity and their close and frequent contact with one another in schools. Antibiotic treatment usually relieves the symptoms of strep throat but fails to irradicate the bacterium 25 to 40% of the time. As a result, 30 to 40% of school children are carriers and able to disseminate the organism to others (Cockerill et al., 1997). Both ill children and healthy carriers are primary streptococcal reservoirs. However, these streptococci are not considered to be members of the normal flora. Although penicillin has been the primary antibiotic prescribed for *S. pyogenes* infections, genetically resistant strains have not been isolated from clinical specimens, a distinction between this species and other Gram-positive streptococci. The basis for persistence of streptococci following vigorous antibiotic therapy may depend on the fact that *S. pyogenes* efficiently invade epithelial cells (LaPenta et al., 1994; Baker and Kasper, 1976), which are impermeable to many antibiotics, including penicillin. This explanation for widespread carriage by children was recently confirmed (Österlund et al., 1997). Tonsils from children troubled by recurrent tonsillitis were shown to harbor *S. pyogenes*, sequestered in parakeratinized epithelial cells. Efforts have failed to discover other animal or insect reservoirs of *S. pyogenes*. Thus, tonsils are the likely reservoir for this common pathogen. Dogs have been suspected to be reservoirs of recurrent infections in families; however, when β-hemolytic streptococci from dogs were further characterized, they were usually group G streptococci and were not, therefore, responsible for recurrent infections in a family unit. Groups C and G streptococci (Baker and Kasper, 1985) also can produce pharyngitis and should be distinguished from *S. pyogenes* to assess the risk for more serious complications.

In the early 20th century, scarlet fever often accompanied throat infections. A red rash, occasionally followed by desquamation of the palms and soles of the feet, characterized these more aggressive infections. The rash is thought to be produced by one of several erythrogenic or pyrogenic toxins, which are also superantigens (Ashbaugh et al., 1998). Erythrogenic toxins SpeA, SpeB and SpeC (streptococcal pyrogenic exotoxins A, B and C) are the best characterized of the more than 12 known *S. pyogenes* superantigens. Genes that encode SpeA and SpeC reside on temperate bacteriophages (Weeks and Ferretti, 1986; Goshorn and Schlievert, 1989), and SpeB is encoded by a chromosomal gene (Bohach et al., 1988). The incidence of scarlet fever has waxed and waned over the past 100 years and may depend on whether the predominant strain that is cycling through a given human population at a specific time expresses the appropriate pyrogenic toxin. The relationship between superantigenicity, red rash and other symptoms of scarlet fever is still unclear.

Streptococcal Impetigo Streptococcus pyogenes skin infections are most common among children who live in the tropics and during the summer among children who live in temperate zones (Bisno and Stevens, 1996c). Pyoderma or impetigo is a highly contagious infection, which is more common among economically disadvantaged children. Lesions are usually limited to the face, arms and legs. The superficial dry, crusted lesion may heal spontaneously and is readily

treated with antibiotics. Although lesions often contain staphylococcus, this form of impetigo is easily distinguished from bulbus impetigo that is caused by S. aureus. Skin lesions often also are accompanied by streptococci in the nose and throat. The serotypes responsible for impetigo are usually different from those that cause strep throat and rheumatic fever (Bessen, et al., 1989; Bessen et al., 1995). The biological basis of this tropism is unknown.

Acute Post-streptococcal Glomerulonephritis
Infections by skin strains can be accompanied by acute streptococcal glomerulonephritis (Wannamaker, 1970; Oliveira, 1997). This potentially serious complication involves the accumulation of immune complexes in the kidney basement membrane, and is characterized by gross hematuria, proteinuria and decreased complement (C3) levels in serum. Anti-DNase B and anti-hyaluronidase titers in patients' sera are elevated. In contrast to rheumatic fever, antistreptolysin O (ASO) titers are low. The incidence of streptococcal-induced glomerulonephritis parallels the incidence of impetigo and/or pharyngitis caused by so-called "nephritogenic serotypes." A latent period of 10 days to 2 weeks from the time of infection precedes the onset of this kidney malfunction. The prevalence of streptococcal acute glomerulonephritis is low in the United States, but more common in other regions of the world, including Egypt, South America, New Zealand and India. There is little agreement on the basis for the nephritogenic potential of some serotypes. However, these serotypes have been shown to express a unique isoform of streptokinase (Ohkuni et al., 1992; Ashbaugh et al., 1998), which was shown to induce pathology in rabbit kidneys similar to that seen in human cases of streptococcal acute glomerulonephritis (Nordstrand et al., 1998). The immunological component of this streptococcal complication is not understood. Immunological cross-reactivity between the glomerular membrane protein, vimentin, and M protein has also been reported (Kraus and Beachey, 1990). However, the significance of tissue reactive antibody to pathology in the kidney also is not understood.

Rheumatic Fever Rheumatic fever is a serious complication of strep throat that may involve multiple organs. Symptoms are first apparent 10–14 days following an active infection. Arthritis, Sydenham's chorea and/or a leaky heart valve are the result of an intense immune response to *S. pyogenes* (Stollerman, 1997; Veasy et al., 1997). The incidence of rheumatic fever varies from 0.5 per 100,000 in the United States to 210 per hundred thousand in India. In parts of Africa

the prevalence is greater than 20 per 10,000 people. In many Third World countries, rheumatic heart disease is the single most common cause of cardiac dysfunction and is a major public concern. Beginning in the 1950s, the institution of throat cultures followed by a 10-day course of penicillin significantly reduced the incidence of rheumatic fever in the United States and Europe. However, regional increases of rheumatic fever in the United States have been reported since the early 1980s. Two outbreaks of rheumatic fever in middle- to upper-income families were observed in Utah (Veasy et al., 1994; Veasy et al., 1997). The relationship between an individual's susceptibility to rheumatic fever, strain of streptococcus, and his or her genetic background is complex (Gibofsky et al., 1998).

Rheumatic fever is autoimmune in nature. Substantial evidence implicates immunological cross-reactivity between streptococcal surface macromolecules and host proteins in the pathogenesis of this syndrome. Immunological cross-reactivity, alone, is unlikely to initiate this disease; however, the role of bacterial or host factors have not been defined. An elevated ASO is a useful diagnostic marker. Throat cultures are often negative by the time symptoms appear. Several manifestations, termed the Jones criteria, are used for diagnosis (Dajani et al., 1995). Genetic predisposition has been suggested by several studies. The association of different ethnic HLA phenotypes with rheumatic fever indicates that the relationship of MHC class to disease is complex. Non-HLA B cell markers have the potential to identify those at risk (Zabriskie, 1995; Kaur et al., 1998).

Superimposed on host genetic susceptibility are environmental and bacterial factors. Outbreaks of rheumatic fever are often associated with a specific serotype. In Utah a serotype (M18 strain) was often cultured from patients (Veasy et al., 1994). This observation prompted some to suggest the existence of rheumatogenic strains of *S. pyogenes* (Stollerman, 1997). All such strains fall into the class I group of serotypes. *Streptococcus pyogenes* displays both proteins and polysaccharide, which mimic mammalian tissue antigens (Cunningham, 1999; Cunningham, 1999). The potential of M protein to induce a tissue reactive immune response is most relevant to the pathophysiology of rheumatic fever. Bessen et al. (1995) found a strong correlation between elevated antibody titers against the class I epitope on M protein and the diagnosis of rheumatic fever. Moreover, monoclonal antibody (mAb 10B6), which defines the class I epitope, reacts with skeletal and cardiac myosins (Quinn et al., 1998). A consensus sequence peptide (RRDL) was found in the C repeats of M protein and in heart and skeletal myosin. Pep-

tides that contain this sequence induce heart-reactive antibody and T-cell responses (Cunningham, 1999). Goldstein et al. (1993) reported that the group A polysaccharide induces antibodies that cross-react with *N*-acetyl-glucosamine glycoprotein in heart valves. Their findings were recently confirmed (Adderson et al., 1998). Most clinical isolates of *S. pyogenes* produce copious amounts of hyaluronic acid, which is biochemically identical to that in human tissue (Bisno and Stevens, 1996c). However, to date little evidence indicates that hyaluronic acid is immunogenic; therefore, it is unlikely to play an important role in the clinical pathology of this autoimmune disease.

The geographic clusters observed in Utah (Veasy et al., 1994, Veasy et al., 1997) are consistent with the possibility that environmental factors increase susceptibility to rheumatic fever. Most cases were from middle income families who received standard medical care. Careful epidemiological studies have not been performed to implicate local differences in medical care, cultural characteristics, air quality, or other microbial infections as possible predisposing influences on susceptibility to rheumatic fever. The genetic homogeneity of Utah residents may be a more important determinant of susceptibility, but appropriate immunogenetic investigations have not been performed.

Invasive Diseases and Toxic Shock An increased incidence of serious streptococcal infections began to be reported in the late 1980s and continued into 2000 (Stevens et al., 1989; Schlievert et al., 1996). Both temporal and geographic clusters of sepsis, toxic shock and necrotizing fasciitis cases have been reported at alarming frequency (Martin and Single, 1993; Martin and Hoiby, 1990). The destructive nature of wound infections prompted the lay press to refer to *S. pyogenes* as "flesh-eating bacteria." Childbed sepsis (puerperal fever) was also reported to be increasing during this period (Colman et al., 1993). The increase in invasive streptococcal disease was associated with emergence of a highly virulent serotype M1 clonal variant (Cleary et al., 1992b), which was disseminated to more than five continents of the world (Musser et al., 1995). The M1 clone (M1inv[+]) produces Spe A (Blumberg et al., 1996) and the cysteine protease (Blumberg et al., 1992), which are thought to be in part responsible for toxic shock and tissue destruction, respectively. Bacterial cells from M1inv[+] cultures were shown to invade epithelial cells at higher frequency than early serotype M1 clonal variants (Baker et al., 1999), prompting Cleary et al. (1998b) to suggest that this trait enhanced persistence and widespread dissemination of the organism. Because clusters of toxic shock were also associated with other serotypes, particularly M3 strains, investigators believe that unidentified host factors may also have played an important role in the resurgence of these dangerous infections (Norrby-Teglund et al., 1994a).

Pathogenesis Streptococcus pyogenes pathogenesis is complex because it depends on a variety of surface-bound macromolecules and extracellular toxins. Infections with disease are not dependent on any one molecule. The species has evolved overlapping mechanisms for adherence to host tissue and multiple, sometimes overlapping, mechanisms to avoid innate host defenses. The application of molecular and genetic techniques to this organism has revealed significant diversity in expression of a wide range of virulence factors. Still a few common themes have become apparent and will be outlined below.

The surface of *S. pyogenes* is covered with a fibrous or hair-like layer, which is composed of proteins and polysaccharides (Fig. 3). This layer appears to substitute for the periplasmic space of Gram-negative bacteria in that many of the proteins are enzymes, such as the C5a peptidase (SCPA), serum opacity factor, glyceraldehyde phosphate dehydrogenase, and peptide transporters. Most surface proteins are anchored to the cell wall by covalent bonds between the peptidoglycan backbone and a LPXTG sequence located at the carboxy-end of each protein (Mazmanian et al., 1999). All clinical isolates express one or more M-like proteins on their surface, C5a peptidase, and one or more fibronectin-binding proteins. Many strains express immunoglobulin, plasminogen and plasmin-binding proteins. *Streptococcus pyogenes* has evolved a surface that mimics host tissue and several mechanisms to avoid or control the cleansing action of the inflammatory response.

Hyaluronic Acid Capsule S. pyogenes surrounds itself with a highly hydrophilic hyaluronic capsule, which contains repeated units of glucuronic acid and *N*-acetyl-glucosamine (Fig. 4). The capsule is biochemically identical to the polysaccharide found in human tissue. Biosynthesis of hyaluronic acid is controlled by the hyaluronic acid synthesis (*has*) operon, which includes three genes (Dougherty, 1994). The *hasA* codes for hyaluronate synthetase (DeAngelis et al., 1993; Dougherty et al., 1994), *hasB* for a UDP-glucose dehydrogenase (Dougherty et al., 1992) and *hasC* for a UDP-glucose pyrophosphorylase (Crater and Van de Rijn, 1995). This UDP-glucose pyrophosphorylase is not required for capsule biosynthesis (Ashbaugh, 1998).

Fig. 3. *Streptococcus pyogenes* surface proteins.
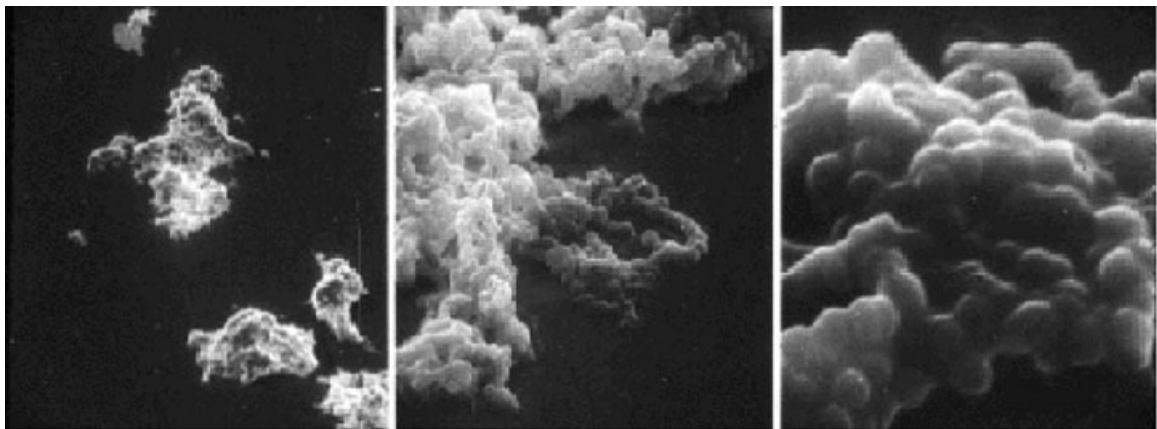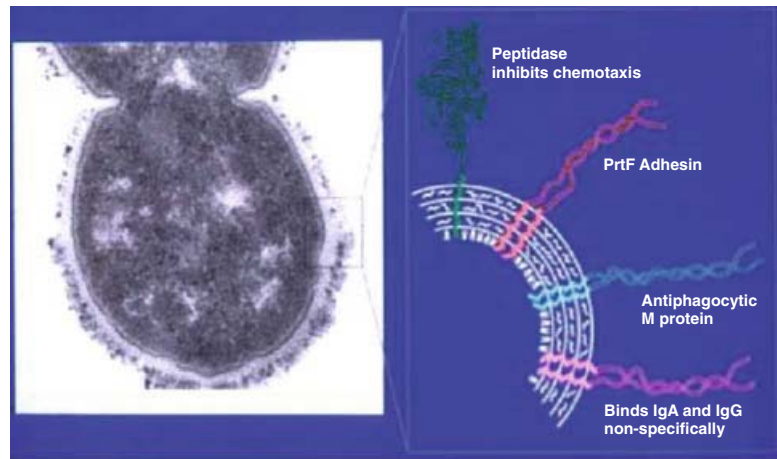
Fig. 4. Scanning electron micrograph of encapsulated *S. pyogenes*. Moderately encapsulated serotype M12 colonies grown on blood agar medium.

Most clinical isolates initially are encapsulated, but the size of the capsule can vary dramatically. Serotype M18 produces such large capsules (Fig. 1) that colonies look like dewdrops. As colonies dehydrate on agar plates, their dewdrop appearance changes to a matte appearance. The "M" for M protein was coined because encapsulated colonies usually also produce M protein (Wilson, 1959). Capsule production is genetically unstable, and phase varies with M protein expression (Cleary et al., 1998c; Ayoub and Swingle, 1985). In serotype M1 strains, the transcription activator, Mga, regulates hyaluronic acid biosynthesis. Because the Mga expression phase varies between on-off states (Bormann and Cleary, 1997), M⁻ colonies also fail to make capsules. Another pair of regulatory genes, *csrR* and *csrS*, was recently identified (Levin and Wessels, 1998; Bernish and van de Rijn, 1999; Heath et al., 1999; Badri et al., 1977). The *csrR* gene encodes a negative regulator that is predicted to be part of a two-component system. Mutations in these or other unidentified loci could give rise to strains, like serotype M18, that produce exceptionally large amounts of hyaluronic acid.

The role of capsule in virulence has been long debated because most cultures of streptococcus produce hyaluronidases, and some body fluids contain hyaluronidases. Recent experiments, however, demonstrated that hyaluronic acid negative mutants are unable to resist phagocytosis or colonize mice (Dale et al., 1996; Wessels et al., 1994). The importance of capsule in disease may depend on the specific strain of *S. pyogenes* and/ or the nature of the organ or tissue that is infected. Capsule was also shown to impede the uptake of oxygen and production of toxic levels of H_2O_2 (Cleary and Larkin, 1979). Therefore, growth in highly oxygenated tissue or on agar plates may select for cultures that produce copious amounts of hyaluronic acid.

C5a Peptidase (SCPA) Streptococcus pyogenes and human isolates of groups B (Bagg et al., 1982) and G streptococci (Baker and Barrett,

1974) express a highly specific serine protease on their surface (Wexler et al., 1985; Hill et al., 1988; Cleary et al., 1991). The SCPA cleaves the human complement protein (C5a) at His^{67}, which is located within the neutrophil-binding site of this chemotaxin (Cleary et al., 1992a). The nucleic acid sequences of peptidase genes, *scpA12*, *scpA49*, *scpA1* and *scpB*, from serotype M12, M49, M1 *S. pyogenes* and *S. agalactiae*, respectively, are available (Chen and Cleary, 1993; Podbielski et al., 1995; Chmouryguina et al., 1996; Cleary, 1998b). Comparisons of these sequences confirmed that peptidases from different streptococci are 95–98% identical. All serotypes tested have the *scpA* gene. Bioassays for residual C5a have been used to measure enzyme activity (Wexler et al., 1985; Ji et al., 1996). A more precise, sensitive fluorometric assay using a recombinant human C5a-GFP (green fluorescent fusion protein) has recently been developed (Stafslien and P. P. Cleary, submitted).

The SCPA is proposed to act at a very early stage of infection, before the bacteria have adapted to the mucosal environment (Fig. 5). Positioned on the outer surface of streptococci, where C5a is produced by activation of the alternative complement pathway, SCPA is poised to destroy the chemotactic gradient as it is formed. Polymorphonuclear leukocytes (PMNs) and other phagocytes, therefore, initially ignore streptococci, providing them an opportunity to colonize (Wexler et al., 1985; Ji et al., 1996). Experiments in mice support this model for SCPA's contribution to streptococcal virulence. In particular, SCPA-deficient mutant streptococci are cleared more rapidly than wild-type bacteria from subdermal (Ji et al., 1996) and mucosal sites of infection (Ji et al., 1998). Mutant

Fig. 5. Model of C5a-peptidase (SCPA) function. Purple spheres represent SCPA$^+$ and SCPA$^-$ *S. pyogenes*. White oblong cells depict polymorphonuclear leukocytes PMNs with pseudopods directed toward SCPA$^-$ streptococci. Blue diamonds represent intact complement C5a chemotaxin and yellow triangles represent cleaved, inactive C5a.

streptococci were also shown to more actively recruit PMNs to sites of infection (Ji et al., 1996). Intranasal immunization of mice induced both SCPA-specific IgA and IgG and increased clearance of streptococci from the nasopharynx of intranasally infected mice (Ji et al., 1997). Interpretation of rodent experiments was questioned by Bohnsack et al. (1993) who were unable to detect destruction of chemotactic activity in zymosan-activated rodent sera. This contradiction has not been resolved as yet. Studies in mice have prompted investigators to begin development of vaccines for protection against both *S. pyogenes* and *S. agalactiae* infections using mutant forms of the enzyme (Baker and Edwards, 1995).

M Protein The M proteins are the most studied products of *S. pyogenes*. Complete or partial DNA sequences of more than 148 *emm* genes have been determined (Beall et al., 1998; Whatmore, et al., 1994; Baker et al., 1976). Two-thirds of the amino-acid sequence, including the carboxyl-amino-acid sequence, is highly conserved and contains the peptidoglycan attachment sequence and a variety of plasma-protein-binding sites (Fig. 6). A 41-amino acid signal sequence is removed during transport to the cell surface to leave N-termini protruding into the aqueous phase. The first 20–30 amino acids of the processed extracellular protein are highly variable, have little structure, and determine serotype specificity. Sequence comparisons of the variable ends revealed that accumulation of amino acid substitutions and small insertions produce variability in antigenic specificity (Harbaugh et al., 1993; Musser et al., 1995). The M proteins are highly a-helical coil-coil structures. A middle rod-like structure is composed of three amino acid repeats termed A, B and C (Fischetti, 1989). Repeat sequences are subject to intragenic recombination, which can also create variation in antigenicity and in the overall size of the protein (Hollingshead et al., 1987). Binding sites for human albumin (Retnoningrum and Cleary, 1994) and serum factor H (Perez-Casal et al., 1995) are located in the C repeats. Fibronectin (Cue et al., 2000), IgG3 (Retnoningrum et al., 1993), and fibrinogen-binding sites overlap the A and B repeats. The hallmark of M proteins is that they confer resistance to phagocytosis on streptococci that display them on their surface. Activation of the alternative complement pathway and deposition of the complement (C3b) opsonin are restricted by M proteins (Jacks-Weis et al., 1982). Horstmann et al. (1992) proposed that factor H, bound to M protein, induces decay of C3b, thereby limiting formation of C3 convertase on the streptococcal surface. This model was challenged by genetic experi-

Fig. 6. M protein architecture.

ments, which showed that deletion of the C repeats eliminated factor H binding to M protein but did not make the mutant streptococcus sensitive to phagocytosis (Perez-Casal et al., 1995). The hypervariable N-terminal segment of M protein was also demonstrated to bind other regulators of complement activation, but the generality of this activity and its role in pathogenesis has not been investigated (Johnsson et al., 1998).

Until recently the M protein was considered to be the primary virulence factor produced by *S. pyogenes* because this protein imparts resistance to phagocytosis in whole blood, the so-called "Lancefield bacteriocidal assay" (1962), and antibody directed against it is opsonic and protective. The importance of M protein for colonization of mice depends on the strain of streptococcus. Thus, M24-deficient mutants were unable to colonize (Courtney et al., 1994), whereas an insertion mutation in the *emm49* gene had little effect on the ability of a serotype M49 strain to colonize mice (Podbielski et al., 1996a; Ji et al., 1998). The M18 protein does not protect the organism from engulfment by phagocytes. Serotype M18 strains depend on a large hyaluronic capsule for resistance to phagocytosis (Dale et al., 1996) and for colonization of mice (Wessels and Bronze, 1994). Other recent experiments have shown that M-protein-dependent resistance to phagocytosis can be overcome when granulocytes are activated by human C5a, lymphocyte function-associated antigen (LFA-1), or an artificial activator, phorbol-12-myristate-14-acetate (PMA; Ji et al., 1998; Schnitzler et al., 1997; 1999). The M proteins are also adhesins and invasins, used by streptococci to invade epithelial cells (Baker and Kasper, 1976; Berkower et al., 1999; Cue et al., 1998; Dombek et al., 1999).

M-like Proteins As geneticists probed the streptococcal chromosome, new surface M-like pro-

teins were discovered. The M-like proteins have an overall architecture resembling that of M proteins, they are co-regulated with M proteins by the Mga transcriptional activator, and their genes are located adjacent to *emm* genes. Extensive sequence similarity between M-like and M proteins suggested that they arose by duplication of an early *emm* gene (Haanes and Cleary, 1989). Some strains produce separate IgG- and IgA-binding proteins (IGP), coded by *mrp* and *enn* genes, respectively (Cleary and Retnoningrum, 1994). These gene designations were changed from their earlier names, *fcrA* (Heath and Cleary, 1989) and *emmL*, respectively. The sequence of *mrp* and *enn* genes from multiple clinical isolates are more conserved than those that encoded M proteins (Podbielski et al., 1994c). However, the distinction between M proteins and M-like proteins has become blurred because M-like proteins also have been discovered to bind immunoglobulins, and IGP proteins have been discovered to also bind fibrinogen (Nilson et al., 1995) and impart resistance to phagocytosis. Both are traditional M-protein characteristics (Podbielski et al., 1996). The range of immunoglobulins bound to IGP proteins has been used to categorize them into groups. Some bind all subclasses of human IgG, whereas others may be restricted to a single subclass. Binding specificity varies with animal species. The most widely recognized streptococcal IGP, protein G, was originally isolated from a strain of group G streptococcus. Protein G is used for affinity purification of IgG and is commercially available (Sigma, Inc.).

Although IGPs have been postulated to be important in virulence, their function in the pathogenesis of *S. pyogenes* is not well understood. Mutations that eliminate M-related protein (Mrp) have little, if any, effect on virulence in mice (Boyle et al., 1998) but are less virulent in a chicken embryo model of infection (Schmidt

et al., 1997). The association of IGPs with the Fc domain of IgG could mimic host tissue by masking the organism with a veil of human immunoglobulin, or by attaching streptococci to antibody-coated surfaces. The IGP proteins could also serve as sponges, which concentrate protein on the streptococcal surface, and could ultimately serve as a source of amino acids. None of these mechanisms are backed by experimental data.

The Geminivirus RepA-binding (GRAB) protein regulates proteolysis at the streptococcal surface. This protein binds α_2-macroglobulin, the primary protease inhibitor in plasma (Rasmussen et al., 1999). Most strains of *S. pyogenes* express GRAB on their surface where it can inhibit streptococcal cysteine protease activity and can protect M protein and SCPA from proteolytic cleavage. Mutations in GRAB significantly decrease virulence in an intraperitoneal mouse model of infection. The GRAB and M-like protein G sequences are related. Groups C and G streptococci also produce α_2-macroglobulin-binding proteins that are presumed to function in a similar manner (Müller and Rantamäki, 1995; Baker and Kasper, 1985).

Plasminogen-binding Proteins Streptococcus pyogenes has evolved multiple proteins on its surface that bind plasmin and/or plasminogen (Lottenberg et al., 1994; Boyle and Lottenberg, 1997). In the presence of plasma, a streptokinase-plasminogen complex is formed that is anchored to the bacterial surface by bound fibrinogen. This complex is an activator of plasminogen and captures the host serine protease (plasmin), which is not regulated by α_2-macroglobulin (D'Costa and Boyle, 1998). The resulting protease activity has been proposed to promote invasion by breaching extracellular matrix barriers to infection. Other surface proteins, glyceraldehyde-3-phosphate dehydrogenase (Winram et al., 1998) and enolase (Pancholi and Fischetti, 1998) also serve as seeds for the accumulation of plasmin on the streptococcal surface.

Extracellular Virulence Factors The cysteine protease (Spe B), also termed "streptococcal pyrogenic exotoxin B," is a major extracellular product of most strains of *S. pyogenes*. This enzyme is expressed as a 371-residue zymogen, which is processed to the 253 residue-active protease (Doran et al., 1999). Tomai et al. (1992) reported that Spe B was a superantigen with the potential to activate $V_\beta 8$ T cells. Gerlach et al., (1983) demonstrated that the cysteine protease and pyrogenic exotoxin Spe B are one and the same molecule. Therefore, both cysteine protease and Spe B designations are found in literature. Biochemical purification of Spe B

revealed contamination with a previously undescribed superantigen, Spe X. This finding and the fact that anti-Spe C or anti-Spe X antibody neutralize the superantigenicity of Spe B prompted Fleischer et al. (1995) to conclude that Spe B (or the cysteine protease) is not a superantigen.

The Spe B molecule has broad substrate specificity and biological activity. Vitronectin and fibronectin are cleaved by Spe B and the interleukin-1β precursor is converted by Spe B to active interleukin-1β, a proinflammatory cytokine (Kapur et al., 1993). The protease was also shown to activate endothelial cell matrix metalloproteases (Burns et al., 1996), which may account for the degradation of extracellular matrix associated with deep tissue infections. In vitro fragments of M protein (Raeder et al., 1998) and C5a peptidase (Berge et al., 1995) are released from the streptococcal surface by Spe B. These observations are difficult to reconcile in light of these surface proteins' importance in virulence. Perhaps the protease functions at a different stage of infection when M protein and the C5a peptidase are no longer required.

Patients who have recovered from both complicated and uncomplicated infections mount an antibody response to Spe B, suggesting that it is expressed during infection. Moreover, Eriksson et al. (1999) presented data that suggested that individuals with lower titers of anti-Spe B antibody were more likely to develop invasive streptococcal disease than those with higher titers. Mutations that inactivate Spe B reduce dissemination of streptococci to organs distant from the site of infection and reduce overall virulence in mouse models (Lukomski et al., 1999). This exotoxin is clearly an important virulence determinant for *S. pyogenes*. The enzyme appears to have very broad substrate specificity, so it is unclear whether cleavage of any of the substrates, identified above, are important to the pathology associated with streptococcal infections.

Superantigens Bacterial superantigens are potent activators of lymphocytes from a variety of mammalian species at concentrations considerably lower than T-cell mitogens, such as concanavalin A. The mechanism of action of these exotoxins was advanced by Marrack and Kappler (1990). Bacterial superantigens, particularly streptococcal toxins, have been the subject of several reviews (Schlievert et al., 1999; Roggiani et al., 1999). They activate specific subsets of T lymphocytes, independent of antigen processing, but require interaction between lymphocytes and macrophages or other antigen-processing cells (APCs). Therefore, activated T cells are not antigen specific but react with a variety of anti-

gens, including "self antigens." Superantigens cross-link the Vb proteins of T cell receptors (TCR) with the invariant region of MHC class II β protein expressed on APCs. The capacity of a particular exoprotein to interact with different TCR-V$_\beta$ proteins determines the subset of T cells, which are activated by that superantigen. Pathology (capillary leakage and liver damage) induced by superantigens is a consequence of a cytokine cascade produced by amplified T cells. Interleukin I, tumor necrosis factor (TNFα) from macrophages, and interferon-γ and TNFβ from T cells mediate the physiological changes associated with toxic shock.

Streptococcus pyogenes secretes a variety of superantigens (Table 4), which were initially termed pyrogenic exotoxins (SPEs) because they induce a distinctive fever and increased sensitivity to endotoxin in rabbits (Watson, 1959). The most studied of the streptococcal superantigens, Spe A, has also been referred to as erythrogenic toxin because it is responsible for the rash associated with scarlet fever. Streptococcal pyrogenic toxins that have been sequenced show from 25–50% identity to each other and to staphylococcal enterotoxins and toxic shock toxin TSST-1. Proft et al. (1999) screened the serotype M1 genome database for a highly conserved small peptide sequence and were able to identify three more superantigens, Spe G, Spe H and Spe J. The three-dimensional structures of Spe A and Spe C were compared to staphylococcal superantigens (Roggiani et al., 1999). They all have common structural features, and Spe A, Spe C and staphylococcus enterotoxin C (SEC), in particular, have a high degree of structural similarity.

Four natural alleles of Spe A were identified among a large number of clinical isolates examined (Nelson et al., 1991). The polymorphism in streptococcal superantigens could account for both quantitative and qualitative differences in the T lymphocytes they activate. Most strains isolated before 1980 produced Spe A1, but the

highly virulent M1inv$^+$ and M3 subclones that emerged some time in the 1980s encode *speA2* and *speA3* alleles, respectively. These each differ from the *speA1* allele by a single different amino acid. The amino acid sequence of a specific superantigen dictates its affinity and V$_\beta$ subset specificity. Kline and Collins (1996a) constructed these allelic forms from a single *speA* gene and analyzed their binding affinity to class II MHC. The *speA3* was found to have a higher affinity, a finding that could explain its greater mitogenic activity. The SMEZ and SMEZ-2 antigens differ by only 17 amino acids, yet they activate expansion of different subsets of T cells (Table 4; Proft et al., 1999).

The literature is replete with descriptions of streptococcal superantigens and the subsets of T cells they activate. However, investigators disagree with regard to the nature of the T cells and cytokines activated by streptococcal exotoxins. Fagin et al. (1997) showed that superantigens purified from streptococci could be contaminated with proteases, DNases, and other superantigens. This has led to misidentification of superantigens and confounded analyses of their activities. They recommend that superantigens be cloned and expressed in *Escherichia coli* and then purified according to their protocol. The V$_\beta$ profiles listed in Table 4 will likely be subject to revision as more pure forms of the proteins are analyzed.

The Spe A and Spe B antigens are encoded by converting temperate bacteriophage. Therefore, the potential to produce these toxins is transmitted horizontally to nontoxigenic strains of *S. pyogenes*. Both *speA* and *speC* genes are located near the phage attachment site, suggesting that the converting phage originated from an abnormal excision by an ancestral phage from a progenitor bacterial chromosome that carried a toxin gene (Goshorn and Schlievert, 1988). More recently, other *speA*-bearing phages have been identified (Yu and Ferretti, 1991). Their size, host range and immune specificity varies. Phage T14

Table 4. Superantigens produced by *S. pyogenes*.

Toxin	T cell subsets activated	Reference
Spe A	Vβ1, 2, 4, 8, 12, 14, 15	Roggiani et al., 1999
Spe B (cysteine protease)	Vβ2, 8	Roggiani et al., 1999
Spe C	Vβ1, 2, 5.1, 10, 15	Tomai et al., 1992
Spe F (DNase)	Vβ2, 4, 7, 8, 15, 19, 21	Norrby-Teglund et al., 1994; Iwasaki et al., 1997
Spe G	Vβ2, 4, 6, 12	Proft et al., 1999
Spe H	Vβ2, 7, 9	Proft et al., 1999
Spe J	ND	Proft et al., 1999
SMEZ-2	Vβ4, 8	Proft et al., 1999
SSA	Vβ1, 3, 5.2, 15	Rede et al., 1999
SMEZ	Vβ2, 4, 7, 8	Kamezawa et al., 1997
SPM-2	Vβ4, 7, 8	Rikiishi et al., 1997
LMWS	Vβ7, 8	Geoffroy-Fauvet et al., 1994

ND, no data.

is harbored by the M1inv[+] clone of streptococcus previously described (Cleary et al., 1998b). Differences in restriction sites suggest that phage T14 has experienced considerable chromosomal rearrangements relative to phage T12, the most studied vector of Spe A. All M1inv[+] strains carry *speA*, but for some unknown reason they produce minuscule quantities of Spe A in vitro.

Epidemiologic surveillance suggests that Spe A plays a pivotal role in toxic shock (Basma et al., 1999). Although only 15% of random, unselected clinical isolates of *S. pyogenes* carry the *speA* gene, highly virulent subclones of serotype M1 and M3, which were associated with the global increase in streptococcal toxic shock and necrotizing fasciitis, uniformly encode this toxin. These same strains also produce Spe B, and a general consensus exists that this protease is responsible for the extensive tissue destruction associated with fasciitis (Schlievert et al., 1999). Little experimental evidence supports this conclusion, however. Purified Spe A induces toxic shock-like changes in rabbits (Lee and Schlievert, 1989) and is lethal for mice (Sriskandan et al., 1996). The lethal dose of Spe A is dramatically decreased when sublethal amounts of lipopolysaccharide (LPS) or endotoxin are administered with Spe A. Schlievert et al. (1996) reported in a review article that immunization of rabbits with Spe A protected them from a lethal challenge with a serotype M3 strain of streptococcus. These results have not been confirmed by others and contradict experiments reported by Sriskandan et al. (1996). The latter found that Spe A did not play an important role in a murine model of fasciitis and multiorgan failure. Moreover, passive immunization with high-titer neutralizing antiserum was not protective. Active immunization with purified Spe A decreased survival following a streptococcal challenge using their model (Sriskandan et al., 1996).

Superantigens are implicated as the primary cause of toxic shock in humans because they induce an array of cytokines in experimental animals similar to those observed in patients suffering from this complication of streptococcal infection. A cause and effect relationship between the superantigenicity activity of these toxins and death in experimental animals is not based on strong experimental evidence. In fact Roggiani et al. (1997) in a genetic analysis of speA produced mutant toxins that (though devoid of superantigenicity) retained toxicity for rabbits. It appears premature to conclude that the superantigenicity of Spe A, or any superantigen produced by *S. pyogenes*, for that matter, is alone directly responsible for toxic shock.

Streptolysin O and S Streptococcus pyogenes produces two hemolysins. The β hemolysis observed on blood agar plates when these streptococci are grown aerobically is primarily dependent on streptolysin S (SLS). Streptolysin O (SLO) is an oxygen-sensitive, pore-forming toxin that is thio-activated (Roggiani et al., 1999). Group C and G streptococci produce closely related hemolysins that are nearly immunogenically, functionally and genetically identical. Nucleic acid sequence and other properties indicate that other bacterial hemolysins, the *S. aureus* α toxin, listeriolysin O (LLO), pneumolysin and perfringenolysin are also closely related to SLO (Tweten et al., 1988; Kehoe et al., 1987). In the past, laboratory preparation of SLO was tedious and met with uncertainty. A new method, which was developed by Gerlach et al. (1993) appears to be a significant improvement.

Regulation of expression of SLO is highly variable and has not been investigated. The *slo* gene product is 571 amino acids, but the mature monomer protein is 46 amino acids smaller (Kehoe et al., 1987). The monomer has four domains, D1-D4. The D4 domain binds to cholesterol, an early, essential step in pore formation. Exogeneous cholesterol inhibits hemolysis in vitro and has been suggested to account for the poor ASO response following skin infections. Bound cholesterol induces a conformational shift in D1 and D3 domains; the shift causes approximately 22 monomers to polymerize and form a pore (Palmer et al., 1998). Target cells then proceed to lyse. A variety of cell types are differentially susceptible to SLO. The high degree of biochemical similarity prompted investigators to test whether SLO and LLO are functionally equivalent hemolysins. Listeriolysin O is required for internalized *Listeria monocytogenes* to escape the phagosome; however, substitution of LLO with SLO did not permit Listeria to disrupt the phagosome and enter the cytoplasm (Portnoy et al., 1992).

The widespread production of SLO-like hemolysins by Gram-positive bacteria is consistent with an important role in their pathogenesis. Although the gene is sequenced and mutant strains have been constructed, we are unaware of animal experiments that confirm this presumption. Streptolysin O is clearly expressed during human infections. In fact, increased titers of anti-SLO (ASO) are a good indicator of streptococcal infection and a hallmark of rheumatic fever. Titers peak 3–6 days following an infection. School children tend to have higher titers than adults. Titers reach a maximum by age 12. Experiments by Ruiz et al. (1998) suggest that SLO, in conjunction with M protein, may regulate the inflammatory response at early stages of skin infection. They examined cytokine responses of cultured keratinocytes, the HaCat cell line, following infection with M[−] and SLO[−]

mutants of *S. pyogenes*. The wild-type culture induced a burst of IL1β, IL6, IL8 and prostaglandin (PGE2) synthesis, whereas mutations in either the *emm* or *slo* genes significantly curtailed this proinflammatory response by keratinocytes. Their results were interpreted to mean that streptococci must be in close contact with target cells to efficiently deliver SLO into the cell's membrane. They also suggested that adhesion to host cells may raise the local concentration of SLO to facilitate polymerization and pore formation. Elevation of IL6 is associated with skin disorders, including psoriasis. Their findings suggested a link between IL6 induction by *S. pyogenes* and the association of these streptococci with acute guttate psoriasis (Ruiz et al., 1998).

Hemolysis on sheep blood agar plates depends primarily on a mysterious, poorly characterized hemolysin, streptolysin S (SLS). This is one of the most potent bacterial hemolysins. Pure toxin has $1 \cdot 10^6$ units of hemolytic activity per mg of protein. A lethal dose of this oxygen-stable hemolysin for mice is 25 µg/kg. Extensive intravascular hemolysis is observed following administration of SLS to rabbits. The toxin causes osmotic lysis of red blood cells (RBCs) and is reported to have a variety of biological activities, but to date no clear picture of its mechanism of action is available. Most strains of *S. pyogenes* and some representatives of other groups of *Streptococci* produce SLS (Alouf and Loridan, 1988). Purification is difficult because SLS is extremely unstable unless complexed with a carrier. Carriers may be albumin, nonionic detergent, trypan blue or RNA. The carrier is purported to be necessary for release of the protein from bacteria (Alouf and Loridan, 1988). The 1,800 Da peptide is produced in late log or early stationary phase and readily decays during prolonged incubation unless serum is present in the growth medium. Higher yields are obtained if growth medium contains an inducer, such as the RNase-resistant fraction of yeast RNA or lipoteichoic acid. An SLS⁻ mutant that was produced by Tn*916* mutagenesis is nonhemolytic on sheep blood agar plates (Nida and Cleary, 1983). It has not been determined whether or not the insertion mutation resides in the toxin gene. Little is known about the genetic control of SLS expression. Mutations in Mga increased production of SLS, suggesting that this transcriptional activator may negatively regulate it (Perez-Casal et al., 1991). Liu et al. identified (by Tn*916* mutagenesis) a genetic locus that is required for SLS expression. Mutations in this locus altered production of other exoproteins and caused a requirement for riboflavin. They proposed that SLS is somehow involved in riboflavin biosynthesis. Betschel et al. (1998) also produced SLS⁻ mutants by Tn*916* mutagenesis. Their mutants were less virulent for mice in a skin infection model. Li et al. (1999) characterized Tn*916* insertion mutants that identified a genetic locus termed "Pel." These mutants were also nonhemolytic. Until the SLS gene has been cloned and sequenced, the mechanism of action of SLS and its role in virulence will continue to be clouded by mystery.

Streptokinase Streptokinase has been referred to as "spreading factor" because it endows streptococci with the capacity to dissolve fibrin clots and then disseminate to surrounding tissue. Most strains of groups C and G streptococci also produce streptokinase protein (Bagg et al., 1982). This highly conserved protein has two polymorphic regions, variable regions V1 and V2 (Huang et al., 1989). Nine different V1 alleles were defined, and *ska1*, *ska2*, *ska6*, and *ska9* were associated with acute post-streptococcal glomerulonephritis (Ohkuni et al., 1992). The bovine pathogen, *S. uberis*, produces a streptokinase that is 25% identical in sequence to those produced by *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis* (Johnsen et al., 1999).

Streptococcus pyogenes goes to great effort to coat its surface with plasmin, which is a highly active protease that can disrupt fibrin clots, degrade basement membrane and other soft tissue glycoproteins. When grown in human plasma, streptococci accumulate proteolytically active plasmin. The presence of plasmin on the surface is thought to allow streptococci to invade and traverse tissue barriers. This organism is armed with one of several plasminogen-binding proteins that can secure either plasminogen or plasmin to their surface (Boyle and Lottenberg, 1997). One such protein, produced by serotype M53 streptococci was termed "PAM," which binds plasminogen in a manner that is resistant to the human regulator of plasminogen activation, α_2AP protein (Ringdahl et al., 1998). Streptokinase then interacts with plasminogen-PAM complexes and induces formation of plasmin. Active plasmin is not formed unless streptococci produce both PAM and streptokinase. Streptokinase-plasminogen complexes have been crystallized and their structure solved (Wang and Zhang, 1998). Streptokinase does not proteolytically cleave plasminogen. Instead, the streptokinase γ domain induces a conformational change in the activation pocket of plasminogen. This change results in autolytic activation to form plasmin. Streptokinase binds to the same segment of plasminogen that interacts with the α_2AP-protein inhibitor; therefore, α_2AP-protein inhibitor no longer controls the activation of plasminogen.

Streptokinase is also thought to be responsible for initiation of acute streptococcal glomerulonephritis (ASGN; Nordstrand et al., 1998; Baker et al., 1999), which damages the kidney glomerular basement membrane. Symptoms include blood and albumin in urine and the deposition of complement C3 protein in the kidney. Acute streptococcal glomerulonephritis follows either throat or skin infections by 10–14 days and does not involve infection of the kidneys. Epidemiologists recognized that ASGN is associated with specific serotypes, such as M2, M49 and M12, to name a few. One of the first clues to suggest that streptokinase may be involved in ASGN was Johnston's recognition that so-called "nephritogenic" strains had common streptokinase alleles that differed from those of non-nephritogenic serotypes (Ohkuni et al., 1992). A previously recognized protein thought to be responsible for ASGN was termed "nephritis strain-associated protein" (NSAP). But NSAP has since been shown to be streptokinase. The concept that streptokinase induces ASGN was supported by Peake et al. (1991) who showed that streptokinase 5, a non-nephritogenic form, has less affinity for isolated glomeruli than a nephritogenic form. They also showed that streptokinase activates the complement pathway. The role of streptokinase in nephritis was confirmed by experiments in which isogenic streptococcal strains that express nephritogenic or non-nephritogenic allelic forms of streptokinase were used in a mouse model of ASGN (Nordstrand et al., 1998). A construct that harbored the *ska1* allele induced symptoms of nephritis, whereas a strain that harbored a *ska5* allele did not. These experiments explained the reason for serotype specificity, but not the reasons for the 10–14 day delay in onset of symptoms and the time required for an antibody response to streptococcal antigens.

Streptokinase is used as a thrombolytic agent for treatment of various blood-clotting disorders, including myocardial infarction (Hoffmeister et al., 1998) and stroke (Levine, 1997). This therapy is complicated by immunological reactions to streptokinase in patients who were previously sensitized by streptococcal infections or therapy with streptokinase. The value of streptokinase and other thrombolytic agents for treatment of these human disorders is still debated.

Adhesins Bacterial pathogens express surface adhesins that interact with receptors on epithelial surfaces of host tissue to resist being washed away by the flow of various body fluids. The specificities of adhesins determine both host and tissue tropisms exhibited by diverse infectious agents. Identification of *S. pyogenes* adhesins has long been a subject of conflict and debate between investigators in the field. This debate has simmered since 1975 when Olfek et al. (1975) published data that suggested lipoteichoic acid (LTA) was the primary adhesin of this species. Controversy has been fueled by many studies that used different strains of streptococci and/or different mammalian cell types. It is now realized that *S. pyogenes* (like other bacterial pathogens) produce multiple adhesins with varied specificities.

Most of the debate was between proponents of the LTA model and those of the M protein model. Ellen and Gibbons (1972) were the first to propose that attachment of streptococci to the oral mucosa of mice is dependent on M protein. Olfek et al. (1975), however, argued that LTA rather than M protein was responsible for streptococcal adherence to buccal epithelial cells. In a recent thorough review of this dispute, Hasty and Courtney (1996) brought the two camps together by proposing a two-step model of attachment. They suggested that LTA loosely tethers streptococci to epithelial cells, and then M protein and/or other fibronectin (Fn)-binding proteins orchestrate a firmer, irreversible association. The importance of Fn-binding proteins was first demonstrated by Talay and her colleagues (Talay et al., 1992). They identified and characterized streptococcal fibronectin-binding protein (Sfb). Shortly thereafter Hanski et al. (1995) characterized an isotype of Sfb, which they termed "protein F." These two groups of investigators have since definitively demonstrated the importance of these proteins in adherence of *S. pyogenes*. Since the discoveries of Sfb and protein F, several distinct Fn-binding proteins have been discovered. Most recently, the M1 and M3 proteins were shown to bind Fn (Cue et al., 1998; Schmidt et al., 1995). The M1 protein was found to be the only Fn-binding protein expressed by the highly virulent M1inv[+] subclone (Cue et al., 1998). This strain was proposed to express at least two adhesins. The primary adhesin for cultured epithelial cells was not identified, but M1-Fn-protein interaction with α5β1-integrin receptors also contributed to the adherence of streptococci to different degrees, depending on the human cell line tested. Protein F-Fn complexes were also shown to direct attachment of M6 streptococci to Hep2 cells via α5β1-integrin receptors (Ozeri et al., 1998).

An important concept that has evolved from these and other studies is that streptococci might employ different adhesins when faced with different physical environments and/or tissues in their host. Strong support for this idea emerged from studies by Okada et al. (1994). In a human skin model of infection, using different isogenic mutants that either expressed M6 protein or protein F, they showed that M protein conferred the potential to adhere to more superficial kerati-

nocytes, whereas protein F directed association with Langerhans' cells. They also showed that the C repeats of M6 protein interacts with the membrane cofactor protein CD46 (Okada et al., 1995). These findings are particularly interesting in light of the fact that M-protein expression is upregulated by CO_2, whereas protein F expression is induced by O_2. Although the concept has not been established, one can easily imagine that both the oxidative potential and the composition of epithelial cells that surround a particular tissue or organ can influence dependence on these different adhesins. The environment, more than likely, changes as infections progress from more superficial tissue to deep-seated or necrotic tissue. With those changes, the adhesive needs of streptococci may also vary.

Intracellular Invasion Until recently, streptococci were considered to be extracellular mucosal pathogens. Group G streptococci were the first discovered to be internalized by epithelial cells, but this aspect of their biology has not been further investigated (Nath, 1989). LaPenta et al. (1994) shortly thereafter demonstrated that *S. pyogenes* efficiently invade the immortalized human lung epithelial cell line A549. Others confirmed their findings using Hep2 (Greco et al., 1995) and keratinocyte cell lines (Schrager et al., 1996). Late log or stationary phase streptococci are internalized at significantly higher frequency than log phase cultures. The standard gentamicin-resistance assay was modified to include penicillin, which, like gentamicin, is unable to kill intracellular bacteria. Some investigators have used low concentrations of Triton X100 to disrupt infected cells, but La Penta et al. (1994) found that even 0.1% Triton X100 decreased the plating efficiency of intracellular streptococci.

Streptococcus pyogenes is generally internalized at relatively high frequency. However, there is considerable differences in frequency depending on the strain and mammalian cells used in the assay. Frequencies range from 10% to 60%. Schrager and Wessels (1996) showed that an exceptionally large hyaluronic acid capsule could impede uptake of streptococci by keratinocytes. Berkower et al. (1999) showed that invasion frequency is influenced by the nature of the M protein expressed by a culture. Cleary et al. (1998c) reported that some cultures are mixtures of high and low invading streptococci, reflecting the genetic instability of M protein expression exhibited by many wild cultures. Serotype M1 clinical isolates were divided into two groups by invasion frequency. The Mlinv[+] subclone is internalized by A549 cells at high frequency, 10–60% of a $1 \cdot 10^5$ colony forming unit (cfu) inoculum, whereas other M1 strains were ingested at significantly lower frequency, less than 0.5%. The clinical source of the M1 strain was irrelevant. Instead, frequency reflected the genotype of the M1 culture (Cleary et al., 1998b).

High frequency invasion of epithelial cells requires that streptococci engage the appropriate integrin receptors on their target cell (Fig. 7; Cue et al., 1998). Adherence alone is not sufficient. Ingestion of Mlinv[+] cultures can be by any one of three different routes. Both Fn and laminin (Lm) can serve as a bridge between the bacteria and their respective integrin receptors to induce endocytosis. The primary invasin for this subclone is the M1 protein, which is a Fn-binding protein. Protein F and SfbI were also demonstrated to be invasins for epithelial cells (Molinari et al., 1997; Ozeri et al., 1998). A third internalizaton pathway is induced by exposing streptococci to small synthetic peptides that con-

Fig. 7. Model of two-receptor-triggered intracellular invasion. Chain of cocci adhering to an epithelial cell via one of several adhesins, which are depicted by a helical dimer. Adherence per se does not trigger ingestion but depends on a fibrinectin (Fn) link between streptococci and the α5β1 integrin on the surface of epithelial cells. The Fn-binding protein of invasin can be either M1 protein, PrF or SfbI.
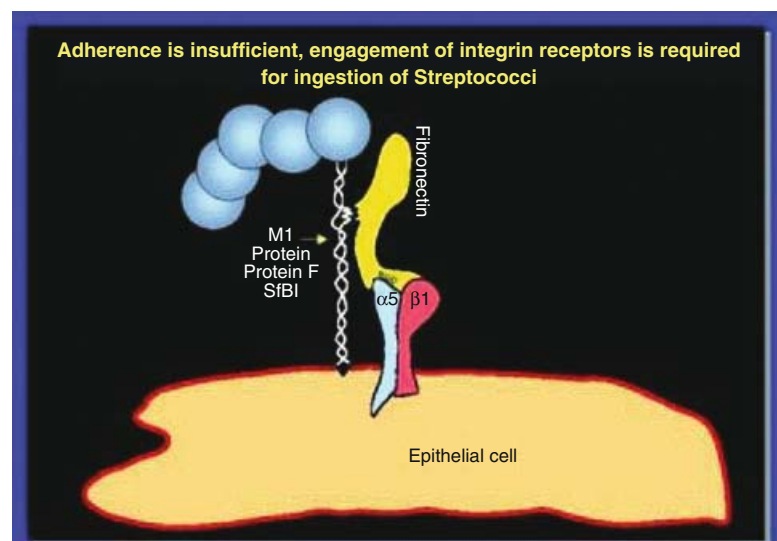
tain RGD sequences (Cue et al., 1998). Fibrinogen was shown to activate invasion of epithelial cells (Cue et al., 1997), but this has since been shown to be due to Fn contamination of commercial preparations of fibrinogen (P. P. Cleary and Q. Cheng, unpublished data).

Streptococci are internalized by receptor-mediated endocytosis into a membrane-bound vacuole. In contrast to *Listeria monocytogenes*, they have not been shown to escape from the vacuole into the cytoplasm, nor have they been shown to multiply while intracellular. Streptococci persist and survive intracellularly for at least a week. Actin polymerization drives endocytic ingestion of streptococci by epithelical cells. Preliminary experiments suggest that some intracellular streptococci are destined to be very rapidly killed, some phagosomes mature into a lyzosome, whereas others are marked to be exported to the cell surface (Dombek et al., 1999).

The correlation between efficient internalization of Mlinv+ streptococci and highly invasive disease associated with this strain of streptococci suggests a cause and effect relationship (La Penta et al., 1994). This observation has not, however, been substantiated. Cleary suggested that intracellular invasion may be more important for dissemination and persistence of streptococci in human populations (Cleary et al., 1998c). Humans are the only known reservoir for this species of streptococcus. Therefore, both immune and nonimmune carriers are likely to be both the reservoir and vehicle that maintains the organism in human populations. Moreover, intracellular streptococci are resistant to penicillin, the most commonly used antibiotic for strep throat. Parents and pediatricians are well aware of children who have recurrent pharyngitis. Children continue to shed streptococci even after a complete course of antibiotic therapy. The best evidence to support a role for intracellular infection and persistent streptococci is from studies of tonsils. Österlund et al. (1997) reported that 13 tonsils from 14 children with recurrent tonsillitis contained intracellular streptococci. They also showed that streptococci could invade keratinized epithelial cells in cultured biopsies of tonsillar tissue. Our laboratory has investigated the capacity of primary keratinized epithelial cells from excised tonsils to internalize M1 streptococci. Invasion of these cells proved to be efficient and dependent on Fn and $\alpha5\beta1$-integrin receptors (Cue et al., 2000). Thus, although it is not yet clear whether intracellular invasion is essential for invasive disease, this characteristic of streptococci appears to promote persistence of the organism in human populations and prevents its eradication by penicillin.

GENETICS

Genetic Variability Like most bacterial pathogens, fresh clinical isolates of *S. pyogenes* and *S. agalactiae* can rapidly change when cultured on laboratory media. Both are genetically unstable and segregate avirulent colonies on storage. Some phenotypic changes, such as hemolysis, mucoidy, or colony opacity are obvious to a suspecting eye. Others surely go unrecognized. Many laboratory cultures are stable presumably because extensive passage has been selected for fast growing colonies that have genetically adapted to growth in artificial media. Virulence genes phase between "on-off" states at frequencies ranging from $1 \cdot 10^{-3}$ to $1 \cdot 10^{-4}$ (Simpson and Cleary, 1987a; Cleary et al., 1998b; Leonard et al., 1998).

Streptococcus pyogenes was first reported to segregate glossy variants from matte or mucoid colonies. The mucoid appearance is dependent on hyaluronic acid, which can become dehydrated to give colonies a matte texture as they age. The matte appearance is also associated with expression of M protein, hence the term "M protein," was coined. Assessment of colony transparency, using a dissecting microscope with obliquely transmitted light can monitor phase variation. The M+ colonies are more opaque, dry and rigid, whereas the M− colonies are more transparent and wet (Simpson and Cleary, 1987a). Colony differentiation requires careful observation because different strains exhibit different and multiple shades of opacity. Although M protein and capsule expression can vary in synchrony, they can also segregate independently. The Mga, itself, phase-varies between "on-off" states, which accounts for the fact that the *emm*, *mrp* and *scpA* phase-vary (Bormann and Cleary, 1997). Transcription of the *speA* exotoxin gene and the *has* operon simultaneously vary with Vir regulon expression in some M1 strains (Cleary et al., 1998c). In these strains, *has* transcription is controlled by Mga, whereas *speA* is not. This finding prompted Cleary et al. (1998b) to suggest that the genetic switch that controls phase variation is part of a more global regulatory circuit. Leonard et al. (1998) reported that older stationary phase cultures produce small colony variants that fail to express many streptococcal products. An intact oligopeptide transport system is required for stabilization of the small colony phenotype (Leonard et al., 1998). The genetic switch that controls variation of virulence factors expression such as M protein has not been identified. The metastable "off" condition may have a selective advantage for intracellular streptococci or for streptococci in a nutrient-poor environment.

Genome Sequence The sequence of the *S. pyogenes* genome is nearly complete. At the time of

this writing it is defined by a single contig, but is still not completely annotated (Beall et al., 1998). The genome is from a serotype M1 strain SF370, which was isolated from a wound infection. This strain is distinct from the highly virulent M1inv[+] clone described by Cleary (1998a) because it lacks a T12-like bacteriophage and the *speA* gene. The method employed involved sequencing random DNA fragments that were produced by shearing and then cloned into a pUC vector. At 1,920 kb, the *S. pyogenes* chromosome is half the size of the *E. coli* genome. A prophage that carries the *speC* gene, a defective phage, IS sequences and transposons has been identified in the genomic sequence. Other categories of genes were recognized that encode regulatory functions, transcription/translation factors, replication functions, energy metabolism and all the previously sequenced virulence factors. The smaller size is consistent with the fastidious nature of this organism. The chromosome is probably lacking genes that encoded for enzymes involved in the biosynthesis of a variety of metabolites. Suvorov and Ferretti (1996) extensively mapped the chromosome using pulse field electrophoresis. Figure 8 provides an overview of the *S. pyogenes* genome (Brandt and Good, 1999). With exception of the M gene, cluster genes that encode virulence factors are dispersed.

Vir Regulon The Vir regulon or M gene cluster varies in size depending on the serotype and strains within a particular serotype. Hollingshead et al. (1993) used this feature to subclassify *S. pyogenes* and to evaluate phylogenetic relationships. Figure 9 shows examples of these differences. The most studied OF[+] class II organism, strain CS101, contains three M-like genes, *mrp*,

emm and *enn* at this locus. For all strains analyzed to date, the cluster is preceded by mga, a positive transcription activator, and is followed by the *scpA* gene. Each gene has its own promoter that contains an Mga-binding site (McIver et al., 1995a). The emm gene clusters of M1inv[+] strains differ from those of earlier M1 strains by the absence of the *prtH* gene, which codes for a second M-like IgG-binding protein. The sic gene encodes an inhibitor of complement lysis. The role of Sic in pathogenesis is unknown, but it is regulated by Mga and is highly divergent in clinical isolates that produce it (Akesson et al., 1996; Stockbauer et al., 1998). The high frequency of amino acid substitutes in Sic suggests that it comes under strong selection and is therefore important in virulence.

Global Regulators Multiple gene activator (Mga) was initially discovered by two groups of investigators (Scott et al., 1995). Robbins and Cleary (1987) designated the gene *virR*, whereas Scott and colleagues referred to it as *mry* (Perez-Casal et al., 1991). The Mga protein is 62 kDa and contains internal sequence homology to phosphate receptor domains of response regulator proteins. For this reason, Mga was suggested to be part of a two-component system. The molecule responds to changes in CO_2 concentration, osmolarity, temperature, free-iron concentration and growth phase (McIver et al., 1995b). McIver et al. (1997) suggested that streptococci monitor these environmental queues as infections progress. Transcription of *mga* is initiated from two promoters and is dependent on Mga itself. Although transcription of *emm* (Perez-Casal et al., 1991; Robbins and Cleary, 1987) and *scpA* are dependent on Mga, their levels of transcription differ (Pritchard et al., 1996). The quantity
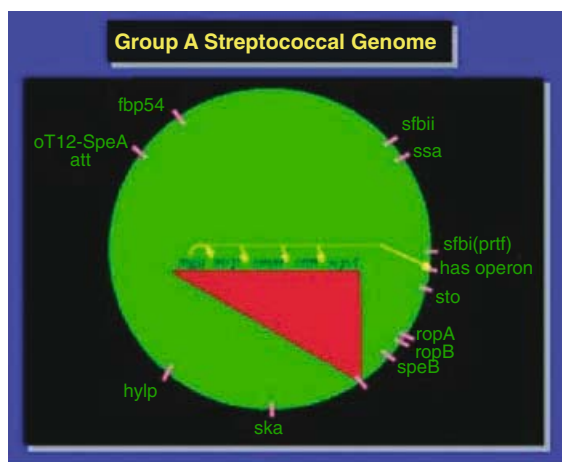
Fig. 8. Map of the *S. pyogenes* genome. This map is a modification of that originally published by Suvorov and Ferreti (1996).
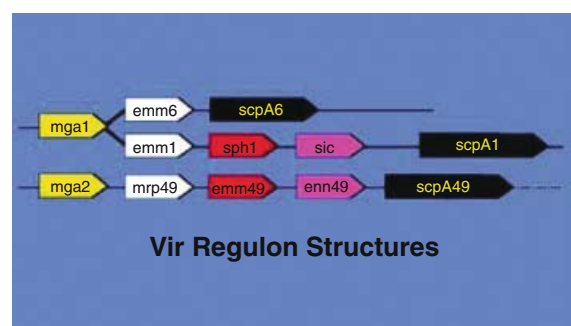
Fig. 9. Vir regulon. Both number of genes and their functions vary. Three examples are shown. The vir regulons from Of[−] M1 and M6 strains usually encode fewer surface proteins than those of Of[+] strains, represented here by an M49 strain. The genes *emm*, *scpA*, *mrp*, *sph* and *enn* genes code for M protein, the C5a peptidase, IgG-binding protein, protein H and IgA-binding proteins, respectively. The *mga* gene codes for the multigene transcriptional activator.

of *scpA* mRNA is relatively constant throughout the growth cycle, whereas *emm* transcription is upregulated in late log to early stationary phase. The *emm* message is also more prevalent. Pritchard et al. (1996) showed that the leader sequence of the *scpA49* gene contains a transcriptional terminator that reduces the concentration of full-length *scpA* mRNA relative to *emm* mRNA. The Mga molecule also regulates genes located outside the M gene cluster. The expression of serum opacity factor, which is expressed by class II serotypes (McLandsborough and Cleary, 1995), hyaluronic acid synthetase (Cleary et al., 1998c), and possibly SLS (Kihlberg et al., 1995) also depend on Mga. The *nra* locus is a negative regulator of genes that encode both fibronectin and collagen binding to the streptococcal surface and also dampens Mga expression. The *nra* gene is self-regulated and responds to growth phase changes (Podbielski et al., 1999).

As noted earlier, some strains of *S. pyogenes* express exceptionally large amounts of hyaluronic acid capsule. Starting with a poorly encapsulated serotype 3 strain, Levin and Wessels et al. (1998) used Tn*916* mutagenesis to produce mutants that are more highly encapsulated. They identified a locus that codes for two proteins, CsrR and CsrS, which resemble two-component regulatory proteins. The CsrR protein was concluded to be a negative regulator of *has* operon transcription.

Two groups of investigators recently discovered a transcription activator of the cysteine protease gene, *speB* (Chaussee et al., 1999; Lyon et al., 1998). This 280-residue protein was termed "RopB." The *ropB* gene maps adjacent to the *speB* gene (Bisno and Stevens, 1996c) and resembles the Rgg activator of an extracellular glucosyl-transferase, which was first identified in *S. gordonii*. Insertional mutations in *ropB* decreased expression of the cysteine protease. Lyon et al. (1998) identified a second gene at the Rop locus that is required for secretion and folding of active protease. The *ropA* gene has homology with trigger factor from *B. subtilis*. This complex protein has peptidyl-prolyl isomerase activity and functions as a chaperone. Podbielski showed that mutations in an oligopeptide transporter also reduced expression of the cysteine protease, suggesting that the peptidase is involved in scavenging amino acids (Podbielski and Leonard, 1998).

In some strains of *S. pyogenes*, the fibronectin-binding protein, PrtF, was discovered to be upregulated by growth in an atmosphere that contains elevated O_2 concentrations. Streptococci grown on the surface of agar plates bind more fibronectin and adhere better to respiratory epithelial and Langerhans' cells (Okada et al., 1994). Transcription of *prtF* depends on the activator *RofA*, which is predicted to be the response regulator of a two-component system. The *rofA* gene is required for its own transcription in an anaerobic environment. Insertional inactivation of *rofA* results in a phenotype where high-level transcription of *prtF* occurs only in response to increased O_2 tension. Expression of *prtF* is constitutive in an anaerobic environment and is controlled by the level of *rofA* transcription (Fogg and Caparon, 1997). Fogg and colleague suggested that control of *prtF* transcription also involves other unidentified regulators.

Genetic Manipulation of S. pyogenes   Identification of virulence determinants of β-hemolytic streptococci progressed as genetic methods for specific inactivation, replacement and for controlled expression of chromosomal genes were developed. Caparon and Scott (1991a) described in detail transformation and conjugation methods. Random transposon mutagenesis was first used in *S. pyogenes* to knock out SLS expression (Nida and Cleary, 1983). This conjugative plasmid encodes tetracycline resistance (tet[r]) and can be crossed into *S. pyogenes* and other species of streptococci by filter mating with enterococci that harbor the transposon. The Tn*916* integrates randomly into the chromosome and occasionally introduces deletions in or near the site of insertion. Experiments can be confounded by multiple insertions of Tn*916* at several locations in the chromosome (Nida and Cleary, 1983; Dale et al., 1996). Proof that changes in phenotype are due to the insertion requires demonstration that reversion to tet[s] is accompanied by return to the original phenotype (Nida and Cleary, 1983). Alternatively, the transposon or tet[r] marker can be transduced by phage A25 from the mutant back into the original parent culture. The parent culture is expected to have acquired the mutant phenotype (Dale et al., 1996). A weakness of Tn*916* mutagenesis is that insertions can be polar on adjacent genes, if those genes are co-transcribed with the gene that contains the insertion. Thus, changes in phenotype must be interpreted with this concern in mind.

As single gene and genomic sequences became available, methods were developed to inactivate specific genes in a directed manner. Most made use of nonreplicating vectors, each containing a homologous fragment that targets insertion into the gene of interest. Several vectors were also constructed to produce merodiploids for complementation experiments (Perez-Casal et al., 1991; Chen et al., 1993) or for expression of different M proteins in a common background (Berkower et al., 1999; Perez-Casal et al., 1992; Courtney et al., 1997; Podbielski et al., 1992). Perez-Casal et

al. (1991) developed methods using the v element. This iterposon encodes kanamycin resistance (kn[r]) and has been used extensively by others (Courtney et al., 1997; Dombek et al., 1999).

Biswas et al. (1993) developed a thermosensitive suicide vector, pG[+]host5, which has been used for gene inactivation and replacement (Fig. 10). This vector contains an erythromycin-resistance gene (erm[r]), an E. coli ori, and a temperature-sensitive streptococcal ori. A fragment of the gene of interest is cloned into a multi-cloning site (MCS) in E. coli. The recombinant plasmid is transformed into S. pyogenes at 30°C with erythromycin selection. The erm[r] streptococcal colonies are then grown in broth at 30°C before being plated out at 40°C on erythromycin-containing media. At 40°C, erm[r] colonies originate from insertion of the plasmid by a single recombination event between the plasmid fragment and homologous sequence in the chromosome. Replacement of the chromosomal gene with that carried by the vector is accomplished relatively easily. Erythromycin-sensitive (erm[s]) revertants can be obtained by transferring stationary phase cultures of the insertion mutant without antibiotic selection. Colonies are then screened for loss of antibiotic resistance. When pG[+]host5 integrates by a single crossover, the fragment of streptococcal DNA is duplicated. Ji et al. took advantage of this feature for construction of gene replacements (Fig. 10; Ji et al., 1997). They replaced a segment of the scpA gene with a fragment that contained a nonpolar deletion in an M49 strain of S. pyogenes. Their approach has an advantage in that the starting culture is not required to first have a selectable marker or deletion in the gene to be replaced. Another advantage of this vector is that it can be easily used with strains that are transformable only at low frequency. The pG[+]host5 system is plagued by at least two technical problems. The plasmid acquires deletions when maintained in E. coli and chromosomal insertions in streptococci are relatively unstable (P. P. Cleary, unpublished observation). Maguin et al. (1996) have updated pG+host5 by adding an IS element. The ISS1 sequence transposes randomly at high frequency. Plasmid pG[+]host5 ends up being flanked by ISS1 copies. Excision of the plasmid leaves a copy of the IS element in the mutated gene.

Podbielski et al. (1996b) constructed a useful set of vectors for insertion mutagenesis and gene replacement for S. pyogenes. Their vectors were

Construction of insertion and deletion mutants of SCPA

1. *scpA* insertion mutants were created by directed plasmid insertion into *scpA*

2. *scpA* deletion mutants were obtained by gene replacement

Fig. 10. Construction of chromosomal mutation using thermosensitive vector pG[+] host5. The gene *erm* codes for erythromycin resistance, *scpA* is the C5a peptidase gene, or a fragment of that gene that contains a defined deletion, indicated by a black triangle. The large X indicates the point of single crossover recombination, which results in insertion of the plasmid into the chromosome. The dashed line shows the position of the recombination event that excises the plasmid from the chromosome. The strain of *S. pyogenes* is CS101sm. This is a modification of a figure published by Ji et al. (1996).
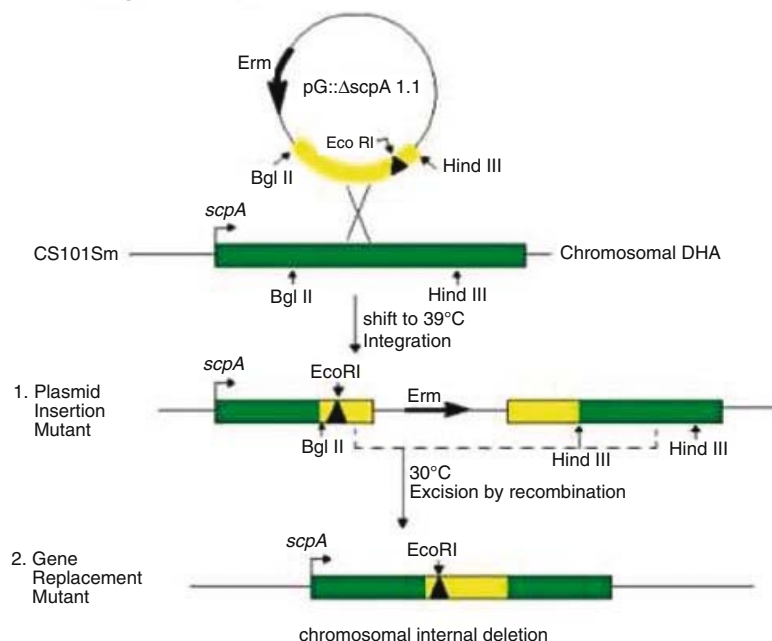
derived from plasmid pSF152. This plasmid has an *E. coli* ori and the spectinomycin gene *aad9*. The copy number is greater than 100 in *E.coli*, but it does not replicate in streptococci. One construct, pFW5, has two MCSs and two terminators. Fragments of the gene to be mutated are cloned into each side of the *aad9* gene. A double crossover leaves the *aad9* gene without the plasmid sequence being inserted in the chromosome, replacing the original gene. Plasmid pFW6 lacks one terminator, so that the inserted *spec^r* gene is not polar on downstream genes. This permits functional analysis of a single gene in an operon. Other variations are plasmids pFW7, 8, 9 and 10, which contain different antibiotic markers (kn^r, cat^r, erm^r, and tet^r, respectively). The antibiotic-resistance gene in each case is constitutively expressed.

Reporter Genes Easy to assay reporter genes, such as chloramphenicol acetyl transferase gene (*cat*), alkaline phosphatase (*phoA*), β galactosidase (*lacZ*), and green fluorescent protein (*gfp*) have been important instruments for unraveling regulatory pathways of a variety of microorganisms. To date only *lacZ* and *cat* gene fusions have found use in *S. pyogenes*. Podbielski et al. (1992), constructed a shuttle vector, pAP, with a staphylococcal cat box for analysis of *emm12* and *scpA* expression in *S. pyogenes*. Pritchard et al. (1996) combined the *emm12::cat* and *scpA::cat* fusions with pG$^+$host5 as a means to construct single copy chromosomal reporters for these genes. The level of CAT expression from Mga fusions was not sufficient to measure Mga expression in streptococci (P. P. Cleary, unpublished observation). Caparon and Scott (1991a) modified the *cat-86* gene from *B. pumilis* and used it to construct mga gene fusions that produced measurable quantities of CAT.

The Tn*917*-LTV*3* transposon, which carries a promoterless *lacZ* gene, also was combined with the thermosensitive pG$^+$host system (Eichenbaum and Scott, 1997). When shuttled from *E. coli* into serotype M6 *S. pyogenes*, the transposon randomly inserted into the chromosome. The *lacZ* gene was positioned so that some insertions created transcriptional fusions, which produced blue colonies on X-gal plates.

Bacteriophage Streptococcus pyogenes is known to be infected by and sensitive to both temperate and virulent bacteriophage. They resemble *E. coli* phage lambda particles morphologically. Their heads contain double-stranded DNA that ranges from 30–40 kb in size. Both types of phage have been used for transduction of antibiotic markers (Malke, 1969; Wannamaker et al., 1973). The virulent phage A25 has most often been used for this purpose (Malke, 1969). Most, if not all, cultures of *S. pyogenes* harbor plaque-forming prophage, which can be induced by UV light or mitomycin (Yu and Ferretti, 1991). Yu and Ferretti found that most strains also conceal a defective phage genome. Detection of phage in clinical cultures is problematic because plaques are usually small, and sensitive indicator strains are often not available. It is usually necessary to test several strains for sensitivity to the phage of interest. Many cultures are infected with multiple phage, and one phage can outgrow the other when production of phage lysates by infection is attempted (Cleary, 1998a). A temperate prophage from a serotype M12 culture was demonstrated to be a cyclic permutation of the vegetative phage genome (Spanier and Cleary, 1983). The genome of this same phage also was found to be terminally redundant, suggesting that it circularizes by homologous recombination within reiterated sequence prior to integration into the streptococci chromosome. The most studied temperate phage, T12, codes for the Spe A exotoxin. This phage integrates by site-specific recombination into a serine tRNA gene creating duplication in that gene (McShan and Ferretti, 1997). The phage T12 (a 35-kb genome) encodes integrase and excisionase-like genes, which are related to those of phage. The phage attachment site is adjacent to the *speA* gene. McShan and Ferretti (1997) have completely sequenced the genome of this phage but have not made that sequence public. These authors also reported that some T12-like phage lacked a *speA* gene, whereas others integrated at bacterial *att* sites other than the tRNA gene. Genomic comparisons by Southern hybridization prompted these investigators to suggest that streptococcal phage with diverse biological and physical properties arise by recombination between functional modules, an idea first put forth by Botstein (1980). This suggestion is consistent with the finding that phage T14 from the highly virulent M1inv$^+$ subclone is a genetically reassorted form of phage T12 (Cleary, 1998a).

A streptococcal-temperate phage also codes for the Spe C exotoxin (Goshorn and Schlievert, 1988), but this phage has not been studied in depth. The temperate phage is also armed to penetrate the extensive hyaluronic acid capsule, which is produced by many strains of *S. pyogenes*. Hynes et al. (1995) have characterized hyaluronidases that are associated with phage particles. It is reasonable to suspect that prophage impact on the biology of *S. pyogenes* in other, yet to be discovered ways, considering the large size of their genomes.

IS Elements and Bacteriocins Insertion sequence (IS) elements are small DNA sequences that may have profound influence on the activity of

adjacent genes or genes into which they insert. They can terminate or activate transcription and can mediate deletion, inversion and duplication of DNA sequences. These mobile elements can flank genes that code for antibiotic-resistance genes or islands of pathogenicity to form composite transposons. They encode transposase enzymes that are essential for transposition from one location to another in the DNA they parasitize. Only two such elements have been described for *S. pyogenes*. Kapur et al. (1994) discovered a 1.1-kb insertion element, IS*1239*. This IS element codes for a putative transposase, which is sequentially related to the *E. coli* IS*30* transposase. A third of the strains they tested harbored IS*1239*, some in multiple copies. The role of IS*1239* in the pathogenesis of *S. pyogenes* was not investigated.

A second IS element was discovered in serotype M1 streptococci by Berge et al. (1998). Insertion element IS1562 is located between sic and *scpA* genes within the M gene cluster. The sequence contains an ORF of 1.2 kb that is probably a transposase and is flanked by three sets of inverted repeats. The IS*1562* sequence is found in the genomic database of *S. pyogenes* and the genomes of serotype M12 and M55 streptococci (Berge et al., 1998). An interesting question raised by the fact that both *emm* and *scpA* genes have been documented to exist in other streptococcal species is whether this IS element defines one boundary of a mobile pathogenicity island in *S. pyogenes*.

Many bacterial genera produce bacteriocins. These extracellular antibiotic-like proteins are usually bactericidal for a narrow spectrum of related bacteria. *Streptococcus pyogenes* bacteriocins recently have been reviewed (Jack et al., 1995). Of these bacteriocins, only SA-FF22 was studied in depth. Tagg and Wannamaker (1978) first identified this lantibiotic-like molecule. The propeptide contains 51 amino acids and the active, mature protein retains one lanthionine and two 3-methyllanthionine residues. The gene that encodes SA-FF22, *scnA*, and adjacent genes are characteristic of a nisin operon (Hynes et al., 1993).

VACCINES Several different approaches to vaccine development for prevention of *S. pyogenes* disease are in progress. Three focus on the M protein, others are directed at the streptococcal C5a peptidase (SCPA), and the group A polysaccharide antigen, which are all bacterial surface antigens. Another is directed at the Spe A and Spe C. The goal of the first five approaches is to induce an immune response that will prevent colonization and infection (Dale et al., 1997). This should, in turn, reduce the overall incidence of *S. pyogenes* infections and also result in fewer cases of more serious infections and complications, such as rheumatic fever and toxic shock.

Intranasal immunization with a genetically inactivated form of SCPA protein imparted immunity against intranasal challenge by multiple serotypes of *S. pyogenes* (Ji et al., 1997; Bisno, 1996a). Immunized mice developed measurable secretory and serum antibodies that are thought to neutralize the enzymatic activity on the surface of infecting bacteria. This surface-bound antibody will enhance clearance of streptococci from the oral-nasal mucosa. Because all serotypes of *S. pyogenes* and groups B, G and C streptococci associated with human infections produce antigenically identical peptidase, vaccines that contain this protein are expected to induce broad protection in humans. More recent experiments have investigated the use of genetically inactivated SCPA, combined with adjuvant, as an injectable vaccine (P. P. Cleary, unpublished data).

The M protein is also the focus of vaccine development by three groups of investigators. Fischetti and collaborators have expressed a fragment of the M protein that contains the conserved C repeats on the surface of *Streptococcus gordonii* (Medaglini et al., 1995). This live vaccine will be used to colonize the oral mucosa of vaccinees. Persistence of the organism is expected to induce a strong protective secretory immunological response. Others have identified a conserved epitope, p145, with the sequence LRRDLDASREAKKQVEKALE in the M protein. Antibodies directed against peptide p145 are opsonic. Aboriginal populations, where *S. pyogenes* infections are endemic, have measurable titers of antibody against this epitope (Brandt and Good, 1999). Dale and his collaborator have turned to the serotype-specific N-terminal end of M protein (Dale, 1999). Their strategy is to construct hybrid M proteins composed of N-terminal sequences from six or more M proteins. Immunization of mice with such multivariant M antigens was shown to induce opsonic antibody against all serotypes represented by the hybrid antigen. The safety of M protein-based vaccines is an important question. The C-repeat region of M proteins has a tissue cross-reactive epitope (Bisno et al., 1997a) and immunization of Lewis rats with M protein induce lesions in the heart that are similar to those seen in humans with rheumatic heart disease (Cunningham, 1999; Bisno et al., 1996b). Massell et al. (1969) immunized a small number of children with M protein and the subsequent incidence of rheumatic fever increased dramatically in those children.

Taxon *Streptococcus agalactiae*

Identification

GROUP B-SPECIFIC ANTIGENS Definitive identification of *S. agalactiae* (group B streptococcus) requires detection of the group B-specific antigen using hyperimmune, grouping antiserum. The group B antigen contains L-rhamnose, D-galactose, 2-acetamido-2-deoxy-D-glucose, and D-glucitol (Michon et al., 1987). It is composed of four different oligosaccharides (designated I, II, III and IV) that are linked by phosphodiester bonds to form a complex, highly branched structure. The original Lancefield method used acid extracts of cell walls as the source of this antigen. Extracts are mixed with grouping serum obtained from rabbits immunized with strain O90R, which is devoid of type-specific antigen. The formation of precipitates in capillary tubes is considered positive. Several rapid methods (presumptive identification) have since been developed for identification of the group B antigen on intact cells, in culture supernatants or bacterial extracts (Facklam et al., 1974; Facklam et al., 1979). Among all the rapid presumptive methods, staphylococcal co-agglutination (Webb et al., 1980) and latex particle agglutination (Ascher et al., 1991) are the methods often used by hospital laboratories (Baker and Edwards, 1995). Researchers have employed both electron microscopy and immunochemical techniques to study the ultrastructure of GBS surfaces (Kasper and Baker, 1979; Wagner et al., 1980). They concluded that the group B antigen traverses the entire cell wall, and the more abundant type-specific capsular polysaccharides appear to mask the group B antigen (Kasper et al., 1978; Kasper et al., 1979).

SUBTYPING GBS The differentiation of group B streptococcal strains into serotypes is based on capsular polysaccharides (type-specific polysaccharides) or cell-wall protein antigens (including α and β antigen, R and X antigens; Table 5; Henrichsen et al., 1984). At present the following serotypes are recognized: Ia, Ib, Ia/c, II, III, IV, V, VI, VII and VIII. The composition of the repeating units of the GBS polysaccharides was determined by methylation analysis combined with gas-liquid chromatography mass spectrometry. Type-specific polysaccharides Ia (Jennings et al., 1983a), Ib (Jennings et al., 1983a), II (Jennings et al., 1983b), III (Wessels et al., 1987), IV (Wessels et al., 1989), V (Wessels et al., 1991), VI (Kogan et al., 1994), VII (Kogan et al., 1995) are high-molecular-weight polymers with a repeating unit composed of glucose, galactose, *N*-acetylglucosamine (except type VI), and *N*-acetylneuraminic acid (sialic acid), which are built from five to seven monosaccharides (Fig. 11). The Type VIII (Kogan et al., 1995) polysaccharide contains a repeating tetrasaccharide unit and a unique rhamnopyranosyl residue. Occasionally, nontypeable strains are isolated from patients and healthy subjects. Despite structural relatedness, the various polysaccharides are immunologically distinct. Countercurrent immunoelectrophoresis and microtiter plate enzyme immunoassays are the most common methods used by reference laboratories for epidemiological studies (Baker and Edwards, 1995). More recently, molecular subtyping methods have been used in epidemiological studies (Baker and Edwards, 1995). They include multilocus enzyme electrophoresis, restriction endonuclease digestion patterns of chromosomal DNA, DNA restriction fragment length polymorphisms (RFLP) of rRNA, pulse field gel electrophoresis, and polymerase chain reaction (PCR) methods. Several subtyping methods have been applied to GBS and suggested that little heterogeneity exists among strains within a given serotype (Blumberg et al., 1992).

Table 5. Protein antigens associated with different serotypes of *S. agalactiae*.

Serotype	Major polysaccharide antigen	Major protein antigens
Ia	Ia	Lmb, β hemolysin, C5a peptidase, CAMP factor
Ib	Ib	C protein, Lmb, β hemolysin, CAMP factor
Ia/c	Ia	C protein, Lmb, β hemolysin, C5a peptidase, CAMP factor
II	II	C protein, R protein, Lmb, β hemolysin, C5a peptidase, CAMP factor
III	III	Rib, R protein, Lmb, β hemolysin, C5a peptidase, X protein, CAMP factor
IV	IV	Lmb, β hemolysin, C5a peptidase, X protein, R protein, CAMP factor
V	V	C protein, Lmb, β hemolysin, C5a peptidase
VI	VI	Lmb, β hemolysin, C5a peptidase, CAMP factor
VII	VII	Lmb, β hemolysin, C5a peptidase, CAMP factor
VIII	VIII	Lmb, β hemolysin, C5a peptidase, CAMP factor
NT		β Hemolysin, CAMP factor

Data was taken from: Spellerberg et al., 1999; Ferrieri, 1988; Flores and Ferrieri, 1989; Edna et al., 1980; Chmouryguina et al., 1996.

Fig. 11. Structure of *S. agalactiae* type III capsular polysaccharide. Data was taken from Baker and Edwards, 1995; Kogan et al., 1994; and 1995.

Multilocus enzyme electrophoresis (MEE) has been used to analyze the population structure of *S. agalactiae* from human sources. Two major clonal groups were identified in strains from the United States (Musser et al., 1989) and France (Quentin et al., 1995). Division I is considered a high virulence clone because the majority of strains were isolated from cases of neonatal invasive disease. Strains from vaginal and rectal carriers were more often placed in division II. Chatellier et al. (1996) investigated the genetic diversity of *S. agalactiae* isolated from the cerebrospinal fluid of neonates and from carriers by RFLP analysis of RNA genes. Two phylogenetic divisions with 40% divergence were observed. The correlation between division and invasive disease is far from absolute but suggests that not all strains isolated from the urogenital tract are able to cause invasive disease. As yet, markers or laboratory characteristics that identify highly virulent strains do not exist.

Disease and Epidemiology

Streptococcus agalactiae (GBS) was historically known as a cause of bovine mastitis until 1938 when it was first recorded to be associated with human infections. It then became appreciated as a serious pathogen of neonates and young infants. Adult disease has emerged more recently and the incidence is rising. As the organism became recognized as a human pathogen, epidemiologic studies examined whether bovine and human strains were biologically related and whether transmission from bovine sources to humans occurs. By comparing culture and physiologic and serologic markers, researchers concluded that transmission of GBS from cattle to human is rare (Jensen, 1996).

The genital and gastrointestinal tracts of many adults are colonized with GBS, yet they remain free of symptoms. The apparent rate of colonization (15–35%) is influenced by body sites

selected for culture, number of cultures obtained from a single site, interval during which specimens are collected, and differences in culture techniques (Baker et al., 1976; Mason et al., 1976; Badri et al., 1977; Gray et al., 1979; Anthony et al., 1981). In a study of 382 patients who were followed by sequential vaginal cultures throughout pregnancy, Anthony et al. (1978) observed that the duration of colonization in any one individual was impossible to predict. Of 108 culture-positive women, 36% had chronic, 20% transient, 15% intermittent and 29% indeterminate carriage of GBS. Hickman et al. (1999) recently studied factors influencing colonization with GBS, including ethnicity and economic status. They observed that colonization was detected in 28% of 546 mothers, was higher in Hispanics (26.9%) and blacks (40.6%) than whites (20.3%) and was not influenced by socioeconomic status. Women, colonized with GBS during pregnancy, are at high risk of premature delivery and prenatal transmission of the organism (Regan et al., 1996).

Group B streptococcus (GBS) is the most common cause of neonatal sepsis and meningitis. Despite antibiotics, GBS initiates a cascade of molecular and biological events in the newborn that leads to altered cerebral perfusion, blood-brain barrier disruption, cerebral edema, intracranial hypertension, neurological damage and even death. It also is associated with chorioamnionitis, endometritis, urinary tract infection and wound infection in pregnant women at the rate of about 10,000 cases per year in the United States. In nonpregnant adults, soft tissue infection, bacteremia, genitourinary tract infection and pneumonia are caused by GBS at a frequency of 2 to 4 cases per 100,000 annually (Farley et al., 1993). An increase of invasive GBS disease in adults was observed in Atlanta (Blumberg et al., 1996). An important feature of the epidemiology of GBS infections is the pronounced effect of age on incidence (Fig. 12). Infections in children are restricted to very early infancy. About 80% of infant infections occur in the first seven days of life, so-called "early-onset disease." Early-onset infection, 1–2 cases per 1,000 births, accounts for 80–85% of all neonatal infections, has a high mortality rate, and is verti-

cally transmitted from colonized mother (Hickman et al., 1999). Vertical transmission of GBS to neonates was significantly diminished when their mothers had intrapartum antibiotics, ruptured membranes >12 hours before delivery, or when the child was delivered by cesarean section (Hickman et al., 1999). Late-onset infections occur in infants between 1 week and 2 to 3 months of age and may be acquired by vertical or horizontal transmission (Weems et al., 1986). Incidence of neonatal GBS diseases during the past 10 years in the United States is summarized in Table 6.

Serotype distribution of GBS infection changes over time. Studies in the 1970s and 1980s indicated that type III isolates might have unique features because they caused the majority of meningitis cases and late-onset infections. This serotype, however, accounts for a smaller percentage of colonizing isolates (Baker and Barrett, 1974; Wenger et al., 1990). During the 1990s, studies observed late-onset diseases among very premature infants and nonpregnant adult disease caused by serotype V, suggesting that this serotype had increased in frequency. In Atlanta, serotype V streptococci caused 21% of all GBS infection. Fourteen percent of early-onset infections and 31% of infection in nonpregnant adults were caused by serotype V bacteria (Blumberg et al., 1996). In Maryland, the predominant serotype, isolated from nonpregnant adults, was serotype V, accounting for 29% of all isolates
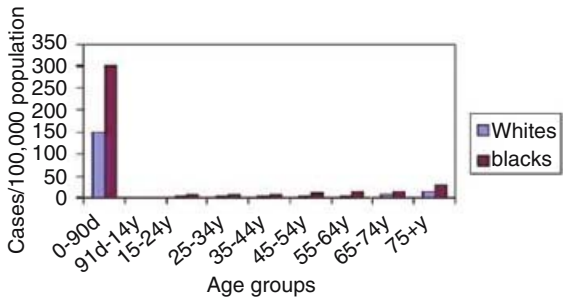
Fig. 12. The incidence of *S. agalactiae* infections is age dependent. Data was taken from the 1993 multi-state population study by the Centers for Disease Control and Prevention (CDC).

Table 6. Ten-year incidence of *S. agalactiae* infections in the United States.

| Population-based study | Date of study | Number of births | Incidence (cases /1,000 births) | | References |
			Early-onset	Late-onset	
Multistate	1990	180,000	1.4	0.3	Zangwill et al., 1992
Multistate	1991–1992	124,464	1.8	ND	Schuchate et al., 1994
Multistate	1993–1995	190,000	1.7	0.5	CDC, 1997
Multistate	1995–1997	97,919	1.1	ND	Lin et al., 1999
Atlanta	1998	45,962	0.73	0.43	Farley et al., 1999

Symbols: ND, no data; CDC, Centers for Disease Control and Prevention.

Neonatal colonization

V 13%
Ia 34%
II 25%
I 28%

Women colonization

V 10%
IV 1%
Ia 35%
II 34%
I 20%

Adult disease

Ib 18%
Ia 6%
I 57%
II 19%

Early-onset Disease

V 12%
V 13%
II 39%
Ia 36%

Late-onset Disease

Ia 23%
II 77%
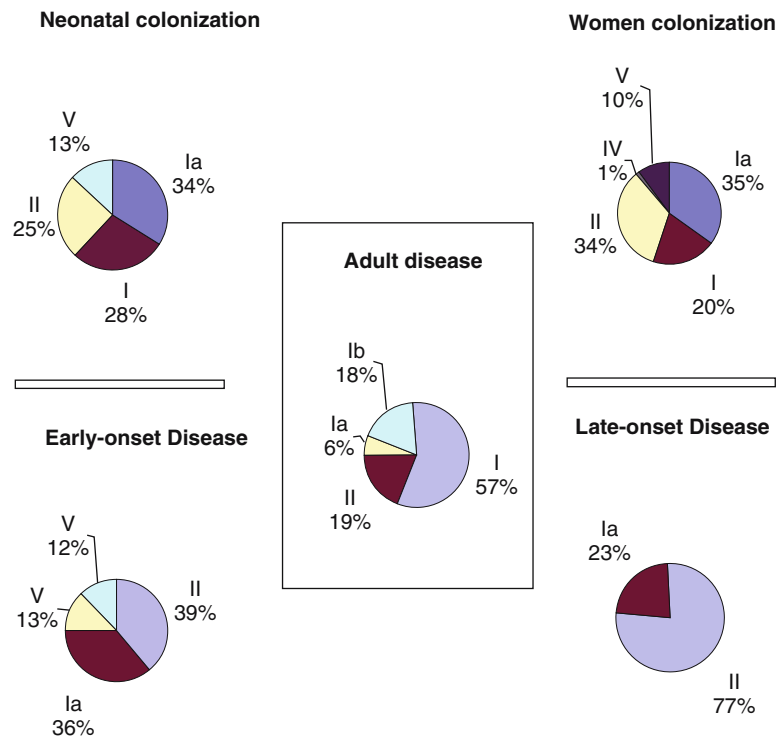
(Harrison et al., 1998). Although not yet commonly recognized within the United States, serotypes VI and VIII frequently were isolated from pregnant women in Japan (Lachenaurer and Madoff, 1996). More recently, a serotype VIII strain was detected among 114 clinical isolates from a Boston hospital (Paoletti et al., 1999).

Characterization of 211 GBS isolates from early-onset disease indicated that serotypes Ia, III and V accounted for 80% of the isolates (Ferrieri and Flores, 1997). Distinctive serotype distributions for different patient groups in the United States are summarized in Fig. 13.

PATHOGENESIS Considerable effort has been made to determine whether GBS from carriers, invasive human disease and cattle have unique, identifiable characteristics. *Streptococcus agalactiae* have been divided into two ecovars. Jensen and Aarestrup (1996) found that 90% of the Danish strains of bovine origin ferment lactose (lac$^+$), whereas 95% of human strains are lac$^-$. Both ecovars, however, may express the same type III capsule polysaccharide. Results of DNA-DNA hybridization and ribotyping experiments also suggested a high degree of relatedness between human and bovine strains (Wanger and Dunny, 1985).

EARLY-ONSET INFECTION The incidence of early-onset GBS infection is only about 1 to 2 cases per 1,000 births, despite the fact that 15–35% of pregnant women are colonized with GBS during pregnancy. Serotypes that were isolated from colonized neonates corresponded to those obtained from their asymptomatically colonized mother (Baker and Edwards, 1995). This suggests that early-onset infections result from ascending spread of the organism into amniotic fluid, followed by aspiration of contaminated amniotic fluid or vaginal secretions containing GBS. Pneumonia may result from local infection, whereas sepsis and meningitis involves spread of bacteria from the lung. Risk factors for early-onset GBS disease include premature delivery, low birth weight, amnionitis with intrapartum fever, heavy colonization of mother, urinary tract infection in pregnancy, rupture of membranes before the onset of labor, prolonged duration of intrauterine monitoring, and low levels of antibodies to type-specific capsular polysaccharides (Schuchat, 1998). Late-onset of GBS infection may also be acquired by horizontal transmission from hospital personnel and other adults.

SURFACE PROTEINS Although several surface proteins have been identified, their role in virulence is not nearly so well defined as those on the surface of *S. pyogenes*. Group B streptococcal strains have trypsin-resistant surface proteins that demonstrate a laddering pattern on sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel. These include a, R (R1 through R5), and Rib proteins (Flores and Ferrieri, 1996; Wastfelt et al., 1997). The regularly spaced ladder pattern is due to hydrolysis of acid-labile Asp-Pro bonds in

repeats that are throughout the sequence of the mature protein (Michel et al., 1992). These proteins were suggested to be members of a family of proteins with related structures (highly repetitive sequence; Wastfelt et al., 1996).

The surface-associated α and β antigens are expressed by many strains of serotype Ia, Ib and II. Trypsin-resistant ladder proteins of serotype V and serotype III share sequence homology with the α antigen (Lachenauerer and Madoff, 1996). The β antigen binds IgA (Russell-Jones et al., 1984; Maeland et al., 1997) but is unrelated antigenically to the IgA receptor (protein Arp) from *S. pyogenes*. Competitive-inhibition binding experiments, however, indicated that they bind to the same region of IgA (Lindahl et al., 1990). The β protein has a higher affinity for serum IgA than for the secretory IgA and binds to the Fc region of these immunoglobulins. Even though the in vivo function of this IgA-binding protein has not been studied, it is assumed that nonimmune binding of IgA would favor mucosal colonization. Rabbit antibodies against both α and β proteins have been demonstrated to confer passive immunity against GBS, expressing the corresponding protein (Lachenauerer and Madoff, 1996).

Neither α nor β antigens are usually expressed by type III strains. A cell surface protein, designated Rib (resistance to protease, immunity, group B), conferred protective immunity in mouse protection assays and is expressed by most strains of type III (31 out of 33 strains; Wastfelt et al., 1997). Antibody directed against the Rib protein does not react with α and β antigens (Stalhammar-Carlemalm et al., 1993). However, the N-termini of Rib and α antigen are 50% identical. Both proteins are resistant to trypsin (Rib is also resistant to pepsin), and both proteins vary greatly in size when different clinical isolates were analyzed.

The R proteins, which are distinct from Rib, are also trypsin resistant, but pepsin sensitive, and can be found in many streptococcal species (Fasola et al., 1996). There are five species of R protein in GBS, and the most frequently encountered R protein is R4 (Flores and Ferrieri, 1989). Linden et al. reported a correlation between low levels of maternal IgG antibodies to R protein and neonatal septicemia with group B streptococci carrying R protein (Linden et al., 1983).

Pattison (1955) discovered the X protein, which is associated with streptococci that cause mastitis in cattle. Experiments showed that cows immunized with purified X protein developed specific antibody and that immune serum could opsonize X-protein-bearing strains in the absence of complement (Rainard et al., 1994).

Different serotypes express unique combinations of these surface proteins. The α antigen was found to be expressed by 43.6% of isolates, making it the most common protein detected on clinical isolates. The R1 and/or R4 proteins were found in 34.6%, α and β in 6.2%, and β and R4 in 1.4%. Each of the common polysaccharide types has a characteristic protein expression pattern: α for Ia, R4 for type III, and R1 and R4 for type V isolates. Expression of α and R proteins are mutually exclusive.

ADHERENCE AND ADHESINS Adherence of group B streptococci to mucosal surfaces presumably represents the first event in colonization and invasion. Group B streptococci adhere efficiently to buccal epithelial cells from infants (Kubin and Ryc, 1988), respiratory epithelial cells (Rubens et al., 1992), endothelial cells (Gibson et al., 1993), macrophage-like cell lines (Cornacchione et al., 1998), primary cultured macrophages (Q. Cheng and P. P. Cleary, unpublished data), vaginal epithelial cells, and to the chorioamniotic membrane (Winram et al., 1998). Winram et al. (1998) recently demonstrated that GBS could invade the chorionic epithelium that surrounds the placenta. They suggested that this could result in inflammatory damage to the amnion, which would permit streptococci to invade the amnionic fluid with subsequent infection of the fetus. Strains from invasive infections of infants adhere more effectively to epithelial cells than to those from age-matched healthy controls (Valentin-Weigand and Chhatwal, 1995). This suggested a strain-dependent difference in adherence, which may impact the incidence of disease. Adhesins have not been identified. Studies suggested that capsular polysaccharides are not involved in adherence and may, in fact, attenuate adherence to some degree (Tamura et al., 1994; Hulse et al., 1993). Lipoteichoic acid (LTA) and surface proteins are potential candidates for adhesins. Investigations of LTA in adherence of GBS are contradictory. Several reports have implicated LTA in adherence by showing that preincubation of epithelial cells with soluble LTA at high concentrations of 500–1,600 µg/ml inhibited attachment of GBS (Nealon and Mattingly, 1984; Cox et al., 1982). Other researchers, however, who employed similar concentrations of LTA did not observe an inhibitory effect (Bagg et al., 1982; Ayoub and Swingle, 1985). Several studies suggested that surface proteins are necessary for adherence by showing that protease treatment reduced adherence and invasion (Bagg, 1982; Hulse et al., 1993). Group B streptococci are also known to have fibronectin- (Tamura et al., 1995) and laminin-binding surface components (Spellerberg et al., 1999). These binding activities may play a role in adherence to epithelial cells. The chemi-

cal environment, such as pH, NaCl and Ca^{2+} and Mg^{2+} concentration, was suggested to influence GBS adherence (Kubin et al., 1983a). Considering that the vaginal pH is less than 4.5, it is not surprising that acidic pH is optimal for adherence (Zawaneh et al., 1979). However, others reported that GBS adherence was optimal at neutral pH (Kubin et al., 1983b), closer to the pH of blood.

INTRACELLULAR INVASION The recently reported ability of GBS to enter and survive in the respiratory epithelial cells may represent a mechanism by which GBS gain access to blood circulation. Because these cells ingested different strains at different frequency, both Rubens et al. (1992) and Valentin-Weigand et al. (1995) suggested that this might account for strain difference in invasiveness. It was also reported that active bacterial protein, DNA and RNA syntheses are required for invasion of epithelial cells. Capsular polysaccharide was not essential for invasion of epithelium (Hulse, 1993). Gibson et al. (1993) reported that GBS was able to invade lung endothelium in vitro and suggested that the ability of GBS to breach the endothelial barrier could provide one mechanism for GBS to gain access to vascular spaces or to enter interstitial spaces from the vascular compartment.

Once GBS invade the host, phagocytic cells (macrophages or polymorphonuclear leukocytes) are required to keep the organism in check. Type-specific antibody is a major opsonin but only promotes phagocytic uptake of homologous GBS strains. Recently, Valentin-Weigand et al. (1996) and Cornacchione et al. (1998) demonstrated that GBS could invade macrophages and persist within them. Prior opsonization with human serum that contains anti-GBS antibodies did not affect bacterial entry but significantly reduced the intracellular survival of streptococci (Valentin-Weigand et al., 1996).

RESISTANCE TO PHAGOCYTOSIS Shigeoka et al. (1978) and Hall et al. (1992) demonstrated that in addition to type-specific antibody, complement is necessary for maximal ingestion of most GBS strains by polymorphonuclear leukocytes (PMNs). Bacterial products that interfere with complement will allow the organism to colonize and persist. Like other encapsulated bacteria, capsule polysaccharides are known to interfere with complement activation by inhibiting C3b deposition. All GBS strains isolated from human sources contain sialic acid as a component of their type-specific capsular polysaccharides. Sialic acid has been shown to be a critical virulence factor of several organisms. It limits C3b deposition by binding factor H, a key regulator of the alternative complement (Marques et al.,

1992). It was demonstrated that the presence and the amount of sialic acid in GBS capsular polysaccharides influenced the incidence of articular lesions, associated with septic arthritis (Tissi et al., 1998).

Other GBS products that may be involved in pathogenesis include C5a peptidase, β hemolysin, and the various surface proteins (α, β, Rib, X, R). The C5a peptidase of GBS (SCPB) is a surface-associated, serine protease (Bisno, 1996a). It cleaves C5a at the residue His-67, a region of the chemotaxin that binds to receptors on the surface of PMNs (Bohnsack et al., 1997). Thus, C5a peptidase will impede clearance of streptococci in several ways: the chemotaxic gradient, which attracts PMNs to the site of infection, is destroyed, and formation of the membrane-attack complex and of other downstream products of the alternative pathway is blocked. Elimination of C5a at infection foci also leaves PMNs in a nonactivated state. Antibodies against C5a peptidase facilitated the killing of GBS by primary bone-marrow-derived macrophage cultures and PMNs (Q. Cheng and P. P. Cleary, unpublished data).

HEMOLYSINS The β hemolysin is suggested to be bound to the bacterial surface. Electron microscopic studies suggested that the β hemolysin is a pore-forming cytolysin (Nizet et al., 1997). Although expression of β-hemolysin activity was correlated with injury of lung epithelial cells in vitro (Nizet et al., 1996), there is little direct evidence for an important role in virulence. Direct damage to host cell membranes could contribute to the severe pneumonia characteristic of early-onset GBS infections. Disruption of epithelial cell barriers also could facilitate access to the bloodstream and systemic spread of GBS. Ninety-eight percent of GBS strains produce the co-hemolysin, termed "CAMP (Christie-Atkins-Munch-Petersen) factor." The *cfb* gene that codes for this streptococcal product was cloned and sequenced by Podbielski et al. (1994a). Lysis by CAMP factor requires that sheep RBCs be simultaneously exposed to the sphingomyelinase β toxin from *Staphylococcus aureus*. Although intravenous inoculation with purified CAMP factor is toxic for rabbits and mice, its actual role in pathogenesis is unknown. Detection of CAMP factor by co-cultivation of *S. aureus* and GBS colonies on the same blood agar plate is a reliable method for identification of GBS.

Genetics

CLONING AND INSERTIONAL MUTAGENESIS To identify GBS virulence factors, a reliable method for cloning genomic genes was developed. Chaffin et al. (1998) developed a cloning

vector pDC123 that allows detection of cells harboring recombinant plasmids when growing on a chromogenic substrate. This vector has a broad host range. Detection of clones relies upon interrupting expression of an *Entercoccus faecalis* alkaline phosphatase (AP) gene, *phoZ*. This reporter functions in both Gram-positive and *E. coli* hosts. The transformation efficiency of GBS is about $1.5–3.6 \cdot 10^5$ cfu/µg of DNA, but varies with the strains employed. Expression of PhoZ in GBS is stable and can be detected after 18–48 hours of incubation. Segregation of *phoZ* was not observed upon serial passage on solid media.

Framson et al. (1997) modified the temperature-sensitive plasmid pWV01 to produce plasmid pTV10K, which contains Tn*917*. This transposon-delivery vector can be used for construction of genomic Tn*917* mutant libraries. They showed that Tn*917* transposed to the GBS chromosome at a frequency of 10^{-3}/cfu and that Tn*917* inserted randomly throughout the chromosome. Our laboratory has used the temperature-sensitive pWV01 plasmid, termed "pG⁺ host 5," for gene replacement (Biswas et al., 1993). A defined deletion was introduced into SCPB gene (Q. Cheng and P. P. Cleary, unpublished data). This deletion mutant is genetically stable and is not complicated by the presence of foreign DNA into the chromosome. We have used this approach to make nonpolar mutations.

INSERTION ELEMENTS An insertion sequence (IS*861*) was identified in the type III strain COH-1 (101). The insertion sequence IS*861* shares greater than 30% homology with IS*3* and IS*150* of *E. coli*, primarily in the region that encodes putative transposases. Multiple copies of IS*861* were observed throughout the chromosome. Granlund et al. (1998) identified a novel insertion element IS*1548*. It was found in hyaluronidase-negative serotype III strains. The insertion sequence IS*1548* was present in 9 of 13 GBS isolates from blood in endocarditis patients. Tamura et al. (2000) cloned an insertion element (IS*1381*) from GBS strain A909, which was previously described in *Streptococcus pneumoniae*. The insertion sequence IS*1381* was found in 18 of 25 unrelated GBS strains. Tamura proposed that patterns of IS*1381* could be an effective tool for subtyping GBS.

Vaccine Development

Development of GBS vaccines began two decades ago when Baker and Kasper (1976b) reported a correlation between maternal antibody deficiency and susceptibility to neonatal GBS infection. Neonate immunity to GBS is associated with naturally acquired maternal antibodies to the type-specific capsular polysaccharides. The IgG antibodies directed against these polysaccharides are passed transplacentally to the fetus. For this reason, the polysaccharide capsules have attracted the most attention for vaccine development. The type III capsular polysaccharide, in particular, has been a focus of these efforts. With the changing serotype distribution and emergence of new serotypes, multivalent polysaccharide-protein conjugate vaccines became an objective. The protein carrier can enhance the immunogenicity of the vaccine by inducing class switching and long-term T-cell memory. Several GBS polysaccharide-protein conjugations are under development. They include tetanus toxoid (Baker et al., 1999), α C protein (Gravekamp et al., 1999), Rib protein (Larsson et al., 1996), β antigen (Madoff, 1994), and SCPB (P. P. Cleary, unpublished data). Larsson et al. (1999) reported development of a GBS vaccine, which contained only protein antigens. This bivalent vaccine is composed of purified Rib and α antigen. Both proteins are expressed by most GBS strains that cause invasive infections. This vaccine was found to protect against the four classical serotypes (Ia, Ib, II and III) in a mouse model.

The initial GBS vaccine was developed for use in pregnant women during the second trimester, after organogenesis was complete, but early enough to permit antibody production before the child is delivered (Baker and Kasper, 1985). The increased invasive GBS disease in nonpregnant adults suggests that the target population for a vaccine should be all women of reproductive age. Some investigators also suggested that a GBS vaccine should be administered with childhood immunizations because vaccine delivery systems in the United States are most efficient at reaching this age group. The conjugate vaccines, which lead to the long-term protection, make this possibility feasible.

Taxon *Streptococcus dysgalactiae*

Taxonomy

Classification of human and animal isolates of group C, G and L streptococci has been confusing, at best, and of little use to microbiologists, at worst. Table 7 lists useful phenotypes that distinguish these groups of streptococci. Biochemical tests and Lancefield grouping sera sort strains into groups that fail to reflect host specificity and common patterns of virulence determinants. The clearest compartmentalization of these organisms is based on DNA-DNA association experiments and MEE (Vieira et al., 1998). Group C, G and L strains were placed into one species, *S. dysgalactiae*. Two subspecies, subsp. *dysgalactiae*

Table 7. Useful characteristics for differentiation of *S. dysgalactiae* strains.

Subgroup	Phenotypic characteristic						
	HEM	GLY	β-D-GAL	HIP	PAHF	BAC	α-L-GLU
S. dysgalactiae subsp. *dysgalactiae*	α	–	–	–	–	S	–
S. dysgalactiae subsp. *equisimilis*							
Human group C	β	–	+	–	+	R	+
Animal group C	β	+	+	–	–	R	+
Human group G	β	–	+	–	+	R	+
Group L	β	+	+	(+)	–	S	+

Symbols: HEM, hemolysis; GLY, acid from glycogen; β-D-GAL, β-D-galactosidase; HIP, hydrolysis of hippurate; PAHF, proteolytic activity on human fibrin; BAC, bacitracin; α-L-GLU, α-L-glutamate aminopeptidase; +, 95% or more of strains positive; (+), 70–80% of strains positive; –, 95% or more of strains negative; S, susceptible; R, resistant.
Adapted from Ruoff (1991).

and subsp. *equisimilis* were defined. The former are α hemolytic and fail to produce β-D-galactosidase and α-L-glutamate amino peptidase; whereas, subsp. *equisimilis* are β hemolytic and produce both enzymes. Subspecies *dysgalactiae*, which react with Lancefield group C antiserum, do not produce the human plasminogen activator, streptokinase, and are often associated with bovine infections. The β-hemolytic subsp. *equisimilis* may express Lancefield C, G or L antigens and streptokinase. With the exception of group L strains, most are sensitive to bacitracin, a characteristic commonly used to identify *S. pyogenes* in clinical laboratories. Human ecovars of subsp. *equisimilis* are very similar, whether they express group C or G antigens. They express related virulence factors on their surface, such as M protein (Bessen et al., 1989), C5a-specific protease (Bessen et al., 1999), and immunoglobulin receptors (Bessen et al., 1995). Group L streptococci of human origin are less studied, but they can also produce immunoglobulin receptors (Sippel et al., 1995). The human ecotypes can be members of the normal flora of throat, skin and vagina, but they also are able to cause infections of consequence.

PATHOGENESIS *Streptococcus dysgalactiae* subsp. *equisimilis* contain group C and G human clinical isolates, and S. equi isolates from horses. Groups C and G are now considered to be agents of human disease. Group C streptococci have been responsible for epidemics of pharyngitis, connected with consumption of contaminants in dairy products. They also have been rarely associated with more severe infection, including bacteremia, acute streptococcal glomerulonephritis, and life threatening invasive disease (Bisno, 1996a). Some investigators question their impact on endemic or community-acquired pharyngitis. Clinicians agree that group C streptococcal pharyngitis may be less severe than that caused by *S. pyogenes* (Bisno et al., 1996b).

Group C and G streptococci can also be a member of the normal flora of the mouth, nose, vagina and intestine, or throats of dogs. Human isolates can be distinguished from those obtained from dogs by the virulence factors they produce. Group C streptococci can mimic the kinds of infections that are more commonly associated with *S. pyogenes*, such as bacteremia, infective endocarditis, osteomyelitis, arthritis and sepsis (Bisno and Stevens, 1996c). Intravenous drug users are particularly prone to group G streptococcal bacteremia. Although some strains express class I M proteins, rheumatic fever is not known to follow infection by this species (Anthony et al., 1981).

M-like Proteins Group C and G streptococci of human origin produce many of the same virulence factors that are expressed by *S. pyogenes*. Simpson et al. (1987b) were the first to demonstrate *emm* genes in group G strains. One strain was demonstrated to have the genetic potential to produce an M12 protein, suggesting that emm genes are horizontally transferred between *S. pyogenes* and group G streptococci. Collins et al. (1992) cloned and sequenced an emm gene, *emmG1*, from a group G streptococcus. The *emmG1* gene encodes a class I type of M protein, yet these streptococci are not associated with rheumatic fever. Southern hybridization analysis of several clinical strains using the *emmG1* gene as a probe suggested that group G streptococcal M proteins might also exhibit serotype variation. Immunochemical analysis of these M proteins has not been performed, so it is not known whether they reflect the serotype specificity of M proteins from *S. pyogenes*. Group G streptococci also produce immunoglobulin-binding proteins. Protein G from this species is used widely for affinity purification of IgG (Fahnestock et al., 1986). Cleary et al. (1991) found that group G streptococci carry C5a peptidase genes that resemble those from *S. pyogenes* DNA. Canine cultures of group G streptococci lacked genes with homology to C5a-peptidase gene probes.

Schnitzler et al. (1997) tested 28 group G streptococci from clinical sources for emm genes

using PCR and primers known to amplify emm genes in *S. pyogenes* DNA. Seventy-five percent of those DNAs encoded M proteins. Bisno and colleagues showed that group C streptococci resist phagocytosis when incubated in human blood. All strains tested harbored *emm* genes, which had extensive homology to those retained by *S. pyogenes* (Bisno et al., 1997b).

Other Virulence Determinants Many putative protein products expressed by these groups of streptococci have been identified as virulence factors by analogy with similar proteins produced by *S. pyogenes*, but none have been studied in depth. Group G streptococci are known to express Fn-binding proteins (Kline et al., 1996b). The role of these proteins in virulence is unknown. It is possible that Fn-binding proteins function as invasins in a manner analogous to *S. pyogenes* (Betschel et al., 1998). Both group C and G streptococci produce streptokinases. The genes that code for this plasminogen activator are highly conserved between species of pathogenic streptococci (Frank et al., 1995).

Human isolates of group G streptococci express C5a peptidase on their surface, which is antigenically similar to that expressed by *S. pyogenes* (Cleary et al., 1991; Bisno, 1996a). The peptidase gene *scpG* was not sequenced, but restriction enzyme mapping suggested that *scpG* and *scpA* are nearly identical. Dog isolates of group G streptococcus do not exhibit C5a peptidase activity, nor do their DNAs contain an *scpA*-like gene. Production of the C5a peptidase is also dependent on whether group C streptococci were isolated from humans or other mammals.

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CHAPTER 1.2.4

Streptococcus pneumoniae

ELAINE TUOMANEN

Introduction

Pneumococcus is a fragile body and contains within itself enzymatic forces that lead to its disruption and disintegration, rob the substrate in which it lives of nutrient substances, and from these substances evolve chemical agents that arrest further growth, cause the death of the organism, and morbidly affect the cells of the animal body into which the microbe may find its way (B. White, 1938).

Streptococcus pneumoniae is currently the leading cause of invasive bacterial disease in children and the elderly. Its original role in causing disease was appreciated by studies on lobar pneumonia in the late 1880s. By the turn of the 20th century, it was determined that antiserum from animals conferred excellent passive protection and even therapeutic benefit to humans stricken with disease. Thus was born the appreciation of the general role of antibodies in host defense and of capsular polysaccharide as a protective target. Antiserum also allowed classification of pneumococci into 2 different types initially (pneumococcus "I" and "Franz"). This was followed by the work of such great microbiologists as Lister, Neufeld, Dochez, Avery and Fleming, who demonstrated an increasing number of serotypes from 4, to 30, and finally to the 90 known today. Later, the natural transformability of pneumococci led to the discovery of DNA as the genetic material (Avery et al., 1944). Thus, both in terms of impact on medicine and on understanding of basic biology, the pneumococcus has been a formidable participant in the advancement of medical science.

Phylogeny

S. pneumoniae are Gram-positive, *a*-hemolytic bacteria. Each bacterium is between 0.5 and 1.25 μ. They have no spores, vacuoles, visible granules or flagella, and they are nonmotile (White, 1938). Like other streptococci, they grow in pairs or short chains, lack catalase and ferment glucose to lactic acid. Unlike streptococci, they do not display an M protein, they hydrolyze inulin, and their cell wall composition is characteristic both in terms of the peptidoglycan and the teichoic acid (alternatively termed C polysaccharide). The pneumococcal cell wall is roughly six layers thick and is composed of peptidoglycan with teichoic acid attached to approximately every third *N*-acetylmuramic acid residue (Garcia-Bustos and Tomasz, 1990; Fig. 1). Lipoteichoic acid is chemically identical to the teichoic acid but is attached to the cell membrane by a lipid moiety. Both the teichoic acid and the lipoteichoic acid contain phosphorylcholine, which has been recognized as an important element in the biology of pneumococcus (Tomasz, 1967). Two choline residues are covalently added to each carbohydrate repeat. The genetic locus for this process, *lic*, has been identified and mutations eliminating the ability to add choline to the surface appear to be crippling or lethal for the pneumococcus (Zhang et al., 1999).

The peptidoglycan is synthesized by a set of at least five cell surface enzymes (penicillin-binding proteins, PBPs; Zighelboim and Tomasz, 1981; Spratt, 1983; Fig. 2). These same constituents bind the β-lactam family of antibiotics by virtue of structural similarity between the antibiotic and the cell wall constituent D-alanyl-D-alanine. These enzymes are responsible for constructing the scaffolding of the wall from intracellular disaccharide pentapeptides, and changes in their affinity for substrate are the molecular mechanism for penicillin resistance. The PBPs are distributed widely on the chromosome, and sequence changes accompanying penicillin resistance are believed to be imported as cassettes from other streptococci by natural transformation (Dowson et al., 1997).

Given the extensive history of pneumococcus in medical research, a sizable library of laboratory strains has been built over the past century (Fig. 3).

Identification

In the clinical laboratory, pneumococci are sought frequently from the respiratory tract,
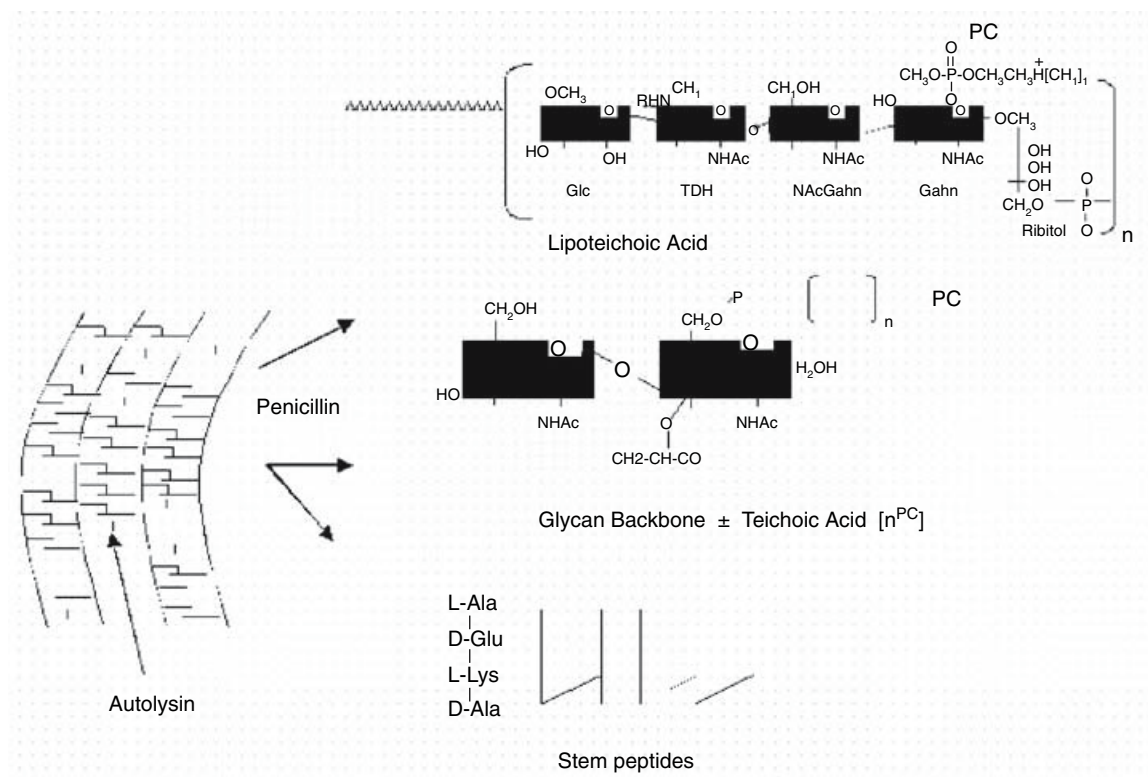
Fig. 1. Structure of the pneumococcal cell wall. The cell wall is composed of a glycan backbone substituted with stem peptides. Approximately every third muramic acid residue is decorated with a complex ribitol containing teichoic acid bearing phosphorylcholine.
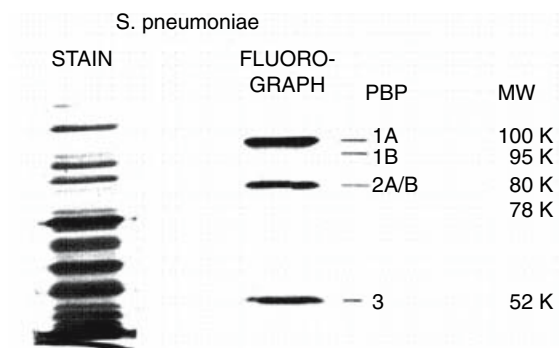

Fig. 2. Comparison of surface proteins and penicillin binding proteins (PBPs). (left) Coomassie blue stain of total surface proteins. (right) Fluorograph of same preparation incubated with 3H penicillin and autoradiographed to demonstrate small subset of proteins devoted to cell wall synthesis. Changes in these PBPs account for β lactam resistance.

middle ear, blood, and cerebrospinal fluid. To facilitate identification of pneumococci in respiratory samples containing multiple species, 5 μg/ml gentamicin or 40 μg/ml nalidixic acid can be added to the medium to suppress the growth of oral flora. The minimum criteria for identifica-

tion and distinction of pneumococci from other streptococci are bile/optochin susceptibility, Gram-positive staining, and hemolytic activity. Pneumococci cause *a*hemolysis on agar containing horse, human, rabbit and sheep erythrocytes (Fig. 4). Under anaerobic conditions they switch to β hemolysis caused by an oxygen-labile hemolysin. Pneumococci form a 16-mm zone around a 5-μg optochin disc and undergo lysis by bile salts (deoxycholate, i.e., the Neufeld phenomenon; Neufeld, 1900). Addition of a few drops of 10% deoxycholate at 37°C lyses the entire culture in minutes. The ability of deoxycholate and penicillin to dissolve the cell wall of the organism depends upon the presence of an autolytic enzyme, LytA (Tomasz et al., 1970). The physiological role of this autolysin is to cause the culture to undergo a characteristic autolysis that kills the entire culture when grown to stationary phase. Virtually all clinical isolates of pneumococci harbor the autolysin and undergo deoxycholate lysis. However, even in the early 1920s it was appreciated that autolysis proceeded at a spectrum of rates, suggesting an underlying variability in this trait (Atkin, 1926). Recently this lack of consistency has been explained by phase variation, a genetically regu-

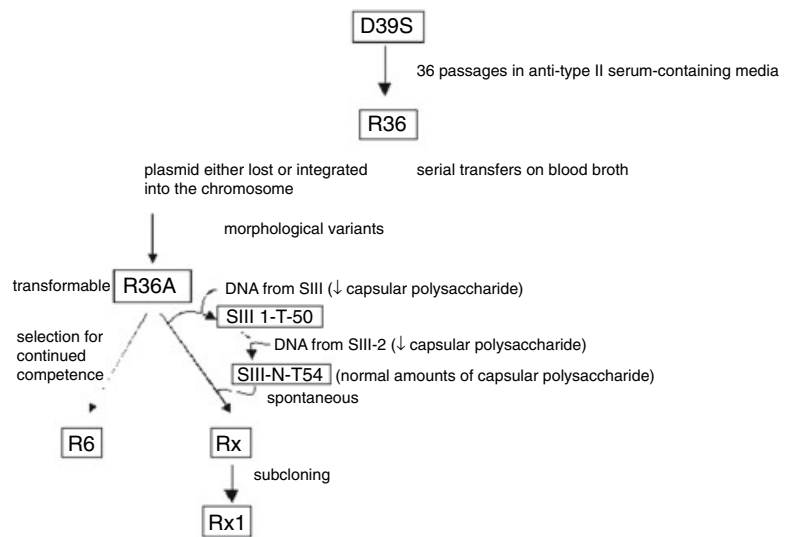Fig. 3. Phylogenic relationship of strains of pneumococcus used in experimental systems in vitro and in vivo.

isolates require fully anaerobic conditions. In all cases, growth requires a source of catalase (e.g., blood) to neutralize the copious amounts of hydrogen peroxide produced by the bacteria. Optimal doubling times of 20–30 minutes require complex media (e.g., brain heart infusion or Todd Hewitt broth, pH 7.8, and incubation at 37°C). Limits for favorable growth are between 25° and 41°C. Dr. Alexander Tomasz (Table 1) has assembled the only chemically defined medium (Cd_{en}) supporting pneumococcal growth. Bacteria can be stored in skim milk or in 10% glycerol at –70°C. Freezing and thawing of a culture leads to a substantial decrease in viability after two cycles.

Fig. 4. Alpha hemolysis characteristic of pneumococci.

On agar, pneumococci grow as 1-mm glistening colonies. Only types 3 and 37 are mucoid. In 1891, Kruse and Pansini first called attention to changes in colonial morphology of pneumococci under in vitro culture and correlated these changes with virulence (Kruse and Pansini, 1891). One hundred years later, this question was revisited by Weiser et al. (1994) who determined that pneumococci spontaneously undergo a genetically determined, phase variation from opaque to transparent colonies at a rate of 1 in 1×10^5 (Fig. 6). The transparent morphotype is adapted to colonization of the nasopharynx, whereas the opaque variant is suited for survival in blood. The chemical basis for the difference in colony appearance is not known, but significant difference in surface protein expression between the two types has been documented.

lated switch between two variants, one expressing large amounts of LytA and the other only small amounts (Weiser et al., 1994).

The Quellung reaction (swelling reaction) forms the basis of serotyping and relies on the swelling of the capsule upon binding of appropriate antibody (Neufeld and Etinger-Tulzunska, 1932; Fig. 5]. The test consists of mixing a loopful of colony with equal quantity of Omniserum (90 types available from Statens Serum Institute, Denmark) and then examining microscopically at 100X for capsular swelling. This effect can be highlighted by the presence of Evans blue in the fluid that is then excluded from the zone around the bacteria by the capsule. Cross-reactivity has been observed between capsular types 2 and 5, 3 and 8, 7 and 18, 13 and 30 and with *E. coli*, *Klebsiella*, salmonella, *H. influenzae* b, viridans streptococci, group B streptococci, and human blood group antigens.

Cultivation

Pneumococcus is fastidious, growing best in 5% carbon dioxide. Some 20% of fresh clinical

Genetics

"Subjected to unfavorable influences, the pneumococcus exhibits great lability of form and function . . . a degraded coccus may develop the
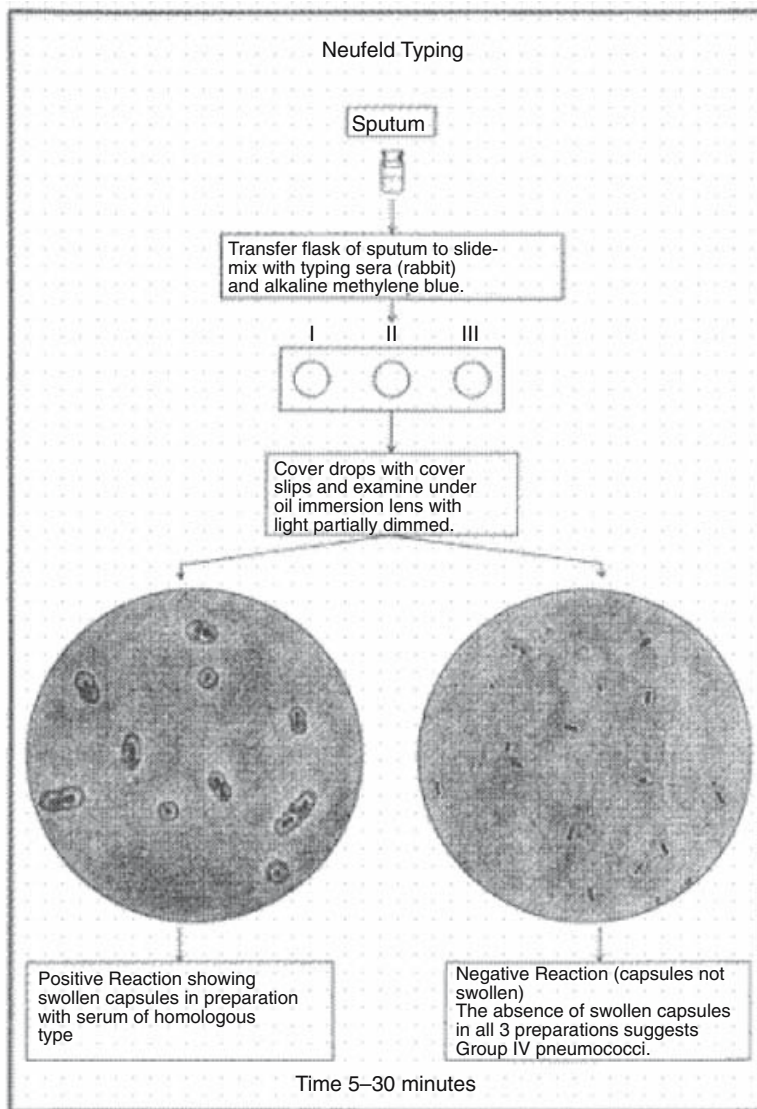
Neufeld Typing

Sputum

Transfer flask of sputum to slide-
mix with typing sera (rabbit)
and alkaline methylene blue.

I   II   III

Cover drops with cover
slips and examine under
oil immersion lens with
light partially dimmed.

Positive Reaction showing
swollen capsules in preparation
with serum of homologous
type

Negative Reaction (capsules not
swollen)
The absence of swollen capsules
in all 3 preparations suggests
Group IV pneumococci.

Time 5–30 minutes

Courtesy of Massachussetts Department of Public Health

CHART 2. THE NEUFELD ON QUELLUNG METHOD OF TYPE DETERMINATION

Fig. 5. Quellung reaction for sero-
typing pneumococci. Capsular swell-
ing in the presence of type specific
antibody is demonstrated by the
halo in the left panel (From White,
1938). This system is still used to
identify the more than 90 different
types of pneumococci.

vital properties of a different specific type. Thus, in addition to natural occurrence of variation . . . the actual transformation of pneumococcal types has been experimentally induced and, possibly, species mutation has occurred." (B. White, 1938).

Such is the description of the natural transformability of pneumococci. Stryker had noted smooth and rough pneumococci, and smooth variants were explained by the production of capsule (Stryker, 1916). Griffith demonstrated that living rough bacteria could acquire the smooth phenotype from killed smooth bacteria (Griffith, 1928). This single experiment subsequently enabled the identification of DNA as the genetic material by Avery, MacCleod, and McCarty (1944). Just over 50 years later

(November, 1997), the nearly complete genomic sequence of *S. pneumoniae* serotype 4, a clinical isolate from a Norwegian boy with meningitis, was placed on-line by The Institute for Genetic Research. A detailed annotation of potential gene products has not been published as yet.

To promote species diversity, *S. pneumoniae* has adopted natural transformation as a mechanism for genetic exchange. This phenomenon is of medical significance because it clearly underlies the explosion of antibiotic resistance in the bacterium over the past 20 years (Munoz et al., 1992). For example, penicillin resistance is due to altered high molecular weight penicillin-binding proteins (PBPs 1a, 2x, 2a, and 2b) which exhibit a low affinity for β-lactam antibiotic (Figueiredo

Table 1. Cd$_{en}$ medium.[a]

Components		Quantity
Add:	Cd$_{en}$ base	200.0ml
	His, Tyr, Arg	50.0ml
	Glutamine (1mg/ml)[b]	10.0ml
	Vitamins w/o choline[b]	10.0ml
	Pyruvate (2% w/v)[b]	5.0ml
	SAC	40.0ml
	Supplement[b]	13.0ml
	KPO$_4$ (1M, pH 8.0)	15.0ml
	Leucine (10mg/ml)	10.0ml
	Phenylalanine (10mg/ml)	5.0ml
	Lysine (10mg/ml)	9.0ml
	Choline (1mg/ml)	2.0ml
	DH$_2$O	26.0ml
Supplement:	3-in-1 salts	60.0ml
	Glucose (20% w/v)	120.0ml
	Sucrose (50% w/v)	6.0ml
	Adenosine (2mg/ml)	120.0ml
	Uridine (2mg/ml)	120.0ml
SAC:	NaCl	12g/liter
	NaOAc (anhydrous)	12g/liter
Cd$_{en}$ base[c]:	Glycine	190mg
	Alanine	350mg
	Valine	720mg
	Isoleucine	760mg
	Proline	1,160mg
	Serine	590mg
	Serine	590mg
	Threonine	450mg
	Methionine	310mg
	Tryptophan	140mg
	Methionine	720mg
	Tryptophan	2,200mg
	Aspartic acid	720mg
	Glutamic acid	2,200mg
	Cysteine	150mg
	q.s. with dH$_2$O	2,000ml
Vitamins w/o choline[b]:	Adam's I	12ml
	Asparagine (5mg/ml)	32ml
	dH$_2$O	36ml
His, Tyr, Arg[b]:	His	640mg
	Try	122mg
	Arg	800mg
	q.s. with dH$_2$O	1,000ml

dH$_2$O, deionized or distilled water.
[a]Filter sterilize after all ingredients are combined.
[b]Filter sterilize.
[c]Adjust to pH 7.0 and filter sterilize.
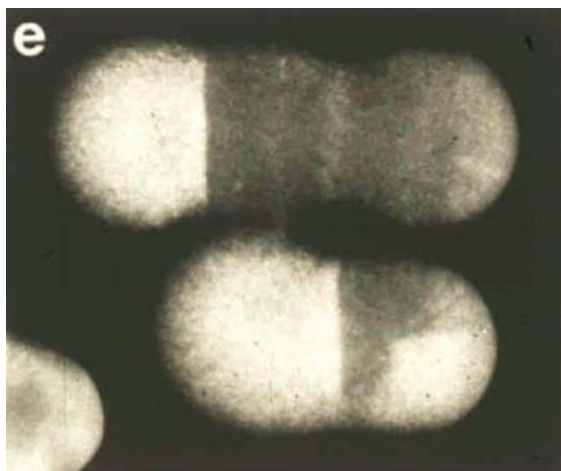From Tomasz (1964).

Fig. 6. Phase variation in colony morphology. Pneumococci vary between opaque and transparent colony morphologies. Opaque are adapted to the bloodstream and transparent to the mucosal surface (from Weiser et al., 1997).

et al., 1992). Sequence analysis of the DNA encoding the PBPs from resistant strains reveals a mosaic gene structure suggesting that cassettes of native PBPs have been replaced by interspecies recombinational events (Dowson et al., 1997). Comparison of the nucleotide sequence of the PBPs between *S. pneumoniae* and *S. mitis* clearly demonstrates horizontal gene transfer.

The binding, uptake, and incorporation of exogenous DNA occur as a sequence of programmed events during a physiologically defined state known as competence (Fig. 7). Competent bacteria self-aggregate, easily form protoplasts, are prone to autolysis and have an increased H$^+$ and Na$^+$ content that leads to increased glycolysis and an enhanced ATP pool. A unique set of at least 11 proteins is preferentially expressed during competence (Morrison and Baker, 1979). This is accomplished by a global regulatory mechanism controlling the competent state (Cheng et al., 1997; Campbell et al., 1998). Early in the competent state, a 17 amino acid peptide, known as competence-stimulating peptide (CSP), is released from the growing bacteria (Havarstein et al., 1995). The CSP has "hormone like" activity in that it induces competence when added to incompetent bacteria following a quorum-sensing paradigm. In addition, CSP is exported through ComA, a member of the ABC family of membrane transporters (Hui and Morrison, 1991). Also in the *com* locus are genes for the two-component system, ComD and E, which serve as the receptor for CSP and the signal transducer that powerfully upregulates gene transcription of the *com* regulon (Havarstein et al., 1996). These downstream genes are characterized by a specific DNA sequence in the promoter and encode proteins that function as the machinery for binding, uptake and recombination of incoming DNA (Campbell et al., 1998). It also has been suggested that healthy pneumococci purposefully release DNA during the competence cycle rather than during autolysis in stationary phase. This is supported by the normal
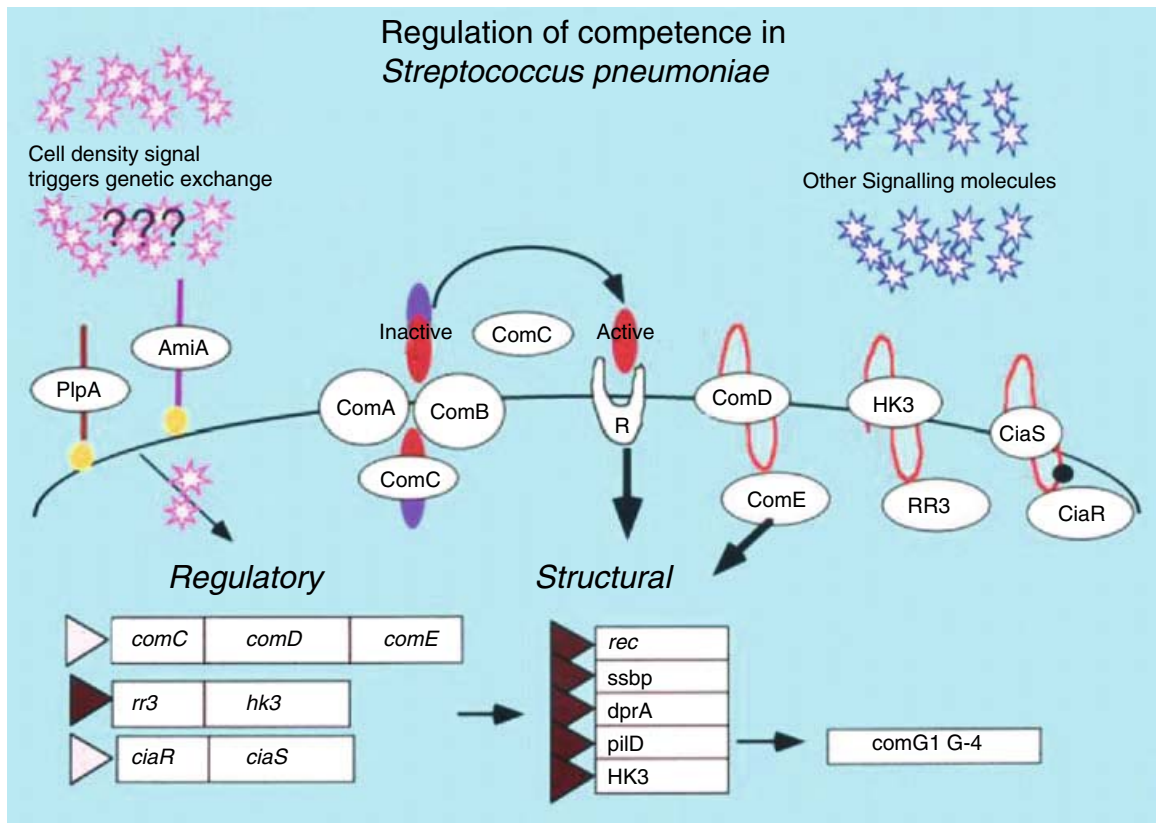
Fig. 7. Regulation of competence for DNA uptake in pneumococci. ComC is a peptide exported by ComA/B, a dedicated transporter. Accumulation of ComC is sensed by a two component system, Com D/E which in turn regulates the transcription of multiple components in the machinery for DNA uptake. This system was the first quorum sensing apparatus described (Tomasz et al., 1970).

competence of strains lacking functional autolysin. A poorly understood process down-regulates competence such that the process is complete in a few minutes during early logarithmic phase.

Epidemiology

S. pneumoniae is a transient commensal, colonizing the nasopharynx of 40% of healthy adults and children with no adverse effects (Gray and Dillon, 1986). Children carry this pathogen in the nasopharynx asymptomatically for about 4–6 weeks, often several serotypes at a time. New serotypes are acquired approximately every 2 months. Serotypes 6, 14, 18, 19, and 23 are the most prevalent accounting for 60–80% of infections depending on the area of the world. Pneumococcal infection accounts for more deaths than any other vaccine-preventable bacterial disease (Gardner and Schaffner, 1993). Those most commonly at risk for pneumococcal infection are children between 6 months and 4 years of age

and adults over 60 years of age. Virtually every child will experience pneumococcal otitis media before the age of 5 years. It is estimated that 25% of all community-acquired pneumonia is due to pneumococcus (1,000 per 100,000 inhabitants). The United States Centers for Disease Control and Prevention (the CDC) reported 60,000 cases of invasive pneumococcal disease in 1997 with ~6,000 deaths. Recently, epidemics of disease have reappeared in settings such as chronic care facilities, military camps and day care centers, a phenomenon not recognized since the pre-antibiotic era. While meningitis is far less common than pneumonia (10 in 100,000 per annum), recent epidemiologic studies suggest that the incidence of meningitis and bacteremia has increased as much as threefold from 1970 to 1990 (Brieman, 1998). Pneumococcal meningitis has a 25% mortality rate, which is higher than that of any other meningeal pathogen (Schuchat et al., 1997). In addition, 50% of the survivors sustain permanent neurological sequelae.

A cause for further alarm is the rapid emergence of multiple antibiotic resistance on a
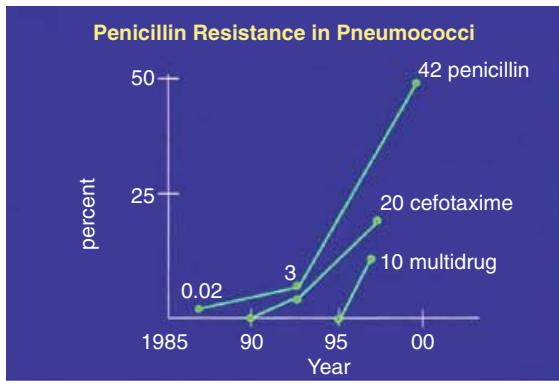
Fig. 8. Increase in antibiotic resistance with time. Increases in penicillin resistance have been followed by resistance to cephalosporins and multidrug resistance rendering this community acquired pathogen progressively difficult to treat.

global scale (Fig. 8). In the early 1970s, it was believed to be a fluke not likely to spread beyond its original sites of origin in Papua New Guinea and South Africa. Multiple antibiotic resistance now covers the globe and has rapidly increased in the last decade so as to complicate greatly the choice of empiric treatment regimens. The prevalence of strains with decreased susceptibility to penicillin has changed the recommended initial antibiotic therapy for childhood meningitis to include vancomycin plus a third-generation cephalosporin. The incidence of resistance to penicillin has increased from <0.02% in 1987 to 3% in 1994 to 30% in some communities in the United States and 80% in regions of some other countries in 1998 (Brieman, 1994). Resistance to other antibiotics has emerged simultaneously: 26% resistant to trimethoprim-sulfa, 9% resistant to cefotaxime, 30% resistant to macrolides, and 25% resistant to multiple drugs (McDougal, 1992). Drug resistance cassettes serve as markers documenting the dissemination of resistant strains from one country to the next, particularly by air travel. Mechanisms vary from changes in the drug target (PBPs for β lactams) to acquisitions of efflux pumps (for macrolides). Acquisition of these properties can occur through transformation of chromosomal determinants or transposition. Conjugative transposons as large as 18 kb (e.g., Tn*916*, *5253*, *1545*, *3701* or *3872*) can carry multiple resistance elements.

Resistant organisms remain fully virulent but seem to have arisen in fewer than 10 serotypes suggesting that some hypervirulent clones have emerged (Enright and Spratt, 1998). Serotypes 6A, 6B, 9V, 14, 19A and 23F cover the vast majority of resistant strains. A second trait creating a background favorable to the emergence of resistance, may be tolerance. In this case, the strain stops growing at the usual concentration

of antibiotic but fails to die. Tolerance was first described in 1970 in laboratory mutants lacking the autolysin LytA, but recently it has emerged in up to 15% of clinical isolates (Tomasz et al., 1970; Handwerger and Tomasz, 1985). The enzyme LytA appears normal in these strains but a regulatory circuit required to initiate LytA autolysis has been downregulated (Novak et al., 1999).

Vaccine

Given the 90 different capsular types of pneumococci, a comprehensive vaccine based on polysaccharide alone is not feasible. Thus, vaccines based on a subgroup of highly prevalent types have been formulated. The number of serotypes in the vaccine has increased from four in 1945 (MacLeod et al., 1945), to 14 in the 1970s (Austrian et al., 1976), and finally to the current 23-valent formulation (25 μg of each of serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F). These serotypes represent 85–90% of those that cause invasive disease and the vaccine efficacy is estimated at 60% (Butler, 1993). However, underutilization is so extensive that the pneumococcus remains the most common agent leading to hospitalization in all age groups (Marrie et al., 1989). This is further complicated by the fact that polysaccharides are not immunogenic in children under the age of 2 years where a significant amount of disease occurs. The polysaccharide vaccine is also not effective for the prevention of common upper respiratory diseases (Klein et al., 1982) and does not reduce nasopharyngeal carriage (Douglas et al., 1986). To address some of these issues, conjugation of a protein antigen to the carbohydrates, thereby generating a T cell-dependent memory response has been undertaken. Phase III trials using a non-pneumococcal protein conjugated to 7, 9 or 11 polysaccharides are under way.

Disease

"The mere presence of these pathogens in the body is insufficient to incite disease; it is the constitutional factors of the host which determine whether the invaders are to remain innocuous . . . [or] act as malignant parasites" (White, 1938).

Pneumococci spontaneously cause disease in humans, monkeys, rabbits, horses, mice and guinea pigs. Nasopharyngeal colonization, pneumonia, otitis media and bacteremia are the most common infections, with meningitis being much more variable. The rabbit and the mouse have been used extensively as animal models of dis-

ease, leading to a reasonable understanding of many of the determinants of pneumococcal virulence.

Capsule

A polysaccharide capsule envelops pneumococcus. Once beyond the mucosal surface, encapsulated strains are 10^5 times more virulent than unencapsulated strains. The capsule interferes with phagocytosis by leukocytes, a property dependent on chemical composition, which engenders differences in site and amount of bound C3b and degradation to iC3d (Hostetter, 1986; Austrian et al., 1976). The capsule does not appear to engage any host defenses except for the generation of antibody-mediated immunity. It also does not interfere with the ability of the underlying components, such as the cell wall and surface proteins, to alert host defense systems. However, C-reactive protein or antibodies to teichoic acid, both of which bind to the cell wall under the capsule, fail to opsonize encapsulated strains. Capsule is gradually shed from the pneumococcal surface in vitro and in vivo, a finding first demonstrated by Preisz (1915).

Cell Wall

In contrast to capsular polysaccharides, the cell wall is a library of potent inflammatory stimuli (Tuomanen et al., 1985; Fig. 9). Challenge with cell wall components alone can recreate many of the symptoms of pneumonia, otitis media and meningitis in experimental models. The phosphorylcholine decorating the teichoic acid and the lipoteichoic acid is a key molecule enabling invasion, and acts both as an adhesin and as a

docking site for cell-surface proteins referred to as choline-binding proteins (see below). Other respiratory pathogens such as *Haemophilus*, *Pseudomonas*, *Neisseria* and *Mycoplasma* also have phosphorylcholine on lipopolysaccharide, proteins or pili suggesting a shared paradigm for the respiratory tract (Weiser et al., 1997). Two host-derived elements that recognize choline are C-reactive protein and the platelet activating factor (PAF) receptor. These respiratory pathogens may be recognized and cleared by the C-reactive protein response of the innate defense system, and they may share invasive mechanisms subverting the signaling cascade of the endogenous chemokine, PAF.

The cell wall directly activates multiple inflammatory cascades: the alternative pathway of the complement cascade generating chemotaxins for leukocytes (Winkelstein and Tomasz, 1978), the coagulation cascade engendering a procoagulant state favoring thrombosis on endothelia (Geelen et al., 1992), and the cytokine cascade inducing interleukin-1β, interleukin 6 and tumor necrosis factor from human cells (Cauwels et al., 1996). Very recently, the activation of these cytokine cascades has been appreciated to occur through toll-like receptor 2 (TLR-2) and CD14 (Yoshimura et al., 1999). Peptidoglycan binds to CD14, a cell surface receptor known to initiate the inflammatory response for endotoxin (Pugin et al., 1994). The TLR-2 then transduces the signal across the cell membrane and initiates a kinase cascade involving (extracellular response kinases) ERKs and p38 mitogen-activated protein kinases (MAPKs) (Schumann et al., 1998) leading to nuclear translocation of a nuclear factor (NF-βB) and induction of cytokine gene transcription (Spellerberg et al., 1996). Contributing to this outcome is the interaction of cell wall choline directly with the G-protein-coupled PAF receptor (Cundell et al., 1995). The earliest manifestation of these activation steps is altered vascular permeability. In lung this leads to a serous exudate, and these effects in the brain result in induction of blood-brain-barrier permeability demonstrable upon intravascular administration of the basic building block of the cell wall, the disaccharide tetrapeptide (Spellerberg et al., 1995). The second phase of the response is heralded by the arrival of leukocytes, thereby making the switch from a serous to a purulent exudate. Sites of pneumococcal infection are particularly noted for the intensity of the purulent response.
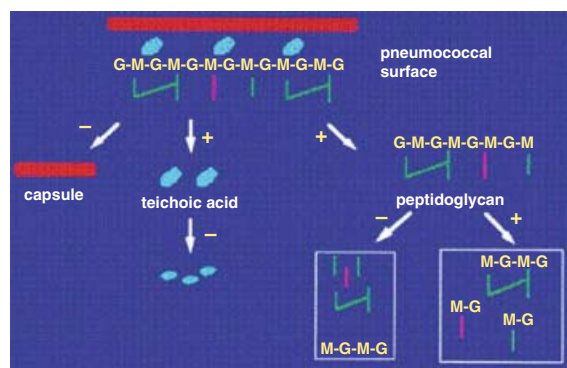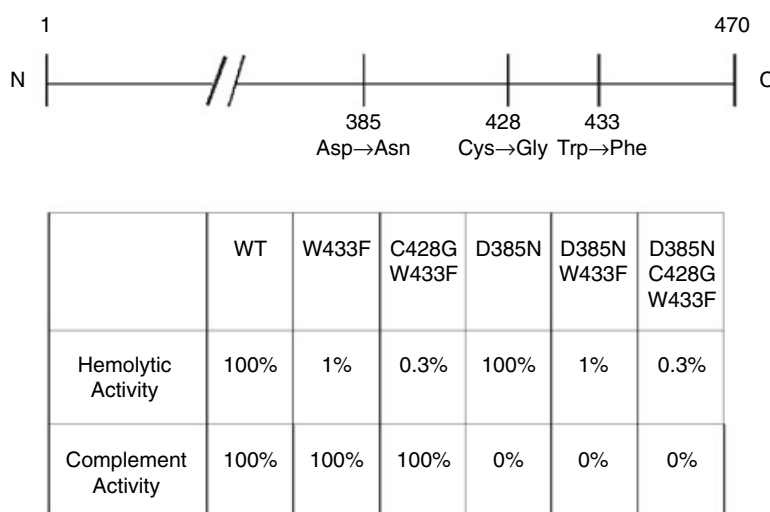

Fig. 9. Inflammatory capacity of pneumococcal surface components. Capsule (red) is noninflammatory. In contrast, the peptidoglycan/teichoic acid complex is highly inflammatory. Smaller components of peptidoglycan progressively lose specific inflammatory activity. This scheme provides a representation of the library of components interacting with host cells during infection.

Surface Proteins

Pneumococci secrete exotoxins and display cell surface-associated virulence determinants. Three

Fig. 10. Schematic representation of bioactivities in pneumolysin.

	WT	W433F	C428G W433F	D385N	D385N W433F	D385N C428G W433F
Hemolytic Activity	100%	1%	0.3%	100%	1%	0.3%
Complement Activity	100%	100%	100%	0%	0%	0%

hemolysins have been described, the most potent of which is pneumolysin, a classical thiol-activated cytotoxin (Mitchell and Andrew, 1997; Fig. 10). Pneumolysin is stored intracellularly and is released upon lysis of pneumococci by autolysin. Pneumolysin binds to cholesterol and thus can indiscriminately bind to all cells without restriction to a receptor. This protein, toxic to nearly every eukaryotic cell, assembles into oligomers resulting in transmembrane pores which ultimately lead to cell lysis. Pneumolysin can also stimulate the production of inflammatory cytokines, inhibit beating of the epithelial cell cilia, inhibit lymphocyte proliferation, decrease the bactericidal activity of neutrophils, and activate complement. The complement activating activity of the toxin has the most impact on virulence in the nasopharynx, whereas both the cytolytic and complement activating activity affect virulence in the lung. The direct effect of the toxin on perfused lungs leads to increased alveolar permeability and abnormalities of the type I alveolar cell. A second hemolysin activity has been described but has not been identified as yet at the genetic level. Finally, pneumococci also produce hydrogen peroxide in amounts greater than human leukocytes produce. This small molecule is a potent hemolysin (Spellerberg et al., 1996).

A combination of genomic analysis of the ~2,000 open reading frames for signal sequences and functional genomics using *phoA* fusion technology to identify exported proteins yields an estimated 500 pneumococcal surface proteins (Pearce et al., 1993). Membrane-associated lipoproteins contain an LXXC motif in the N-terminus that serves as a cleavage site and a covalent-binding site for palmitic acid. There are ~20 proteins containing this motif in the pneumococcal genome, including several peptide permeases and two neuraminidases (Dintilhac

et al., 1997; Novak et al., 1998; Pearce et al., 1994). Proteins are also physically associated with the cell wall by two mechanisms; one covalent and one noncovalent. As many as 15 proteins use a well-characterized LPXTGE motif, which is a cleavage site for a sortase that creates a covalent attachment of the exported protein to the cell-wall stem peptide (Schneewind et al., 1995; Mazmanian et al., 1999). The IgA protease is one example of a member of this family (Wani et al., 1996).

The most unique group of proteins on the pneumococcal surface is the family of choline-binding proteins (CBPs; Fig. 11). Twelve CBPs are noncovalently bound to the choline moiety of the cell wall, thereby reversibly snapping diverse functional elements onto the bacterial surface (Gosink et al., 1999). This is a novel mechanism of attachment for virulence determinants. The CBPs all share a common C-terminal choline-binding domain that consists of 2 to 10 repeats of 20 amino acids (Garcia et al., 1998). The N-termini of the CBPs are distinct indicating their functions are diverse. The CBPs can be adducted onto and off of the choline rapidly by various regulatory mechanisms allowing a repopulation of the surface and a changing of the function of the surface. These mechanisms include phase variation and two-component regulatory systems. For instance, reversible phase variation of colony morphology from opaque to transparent changes the amounts of capsular material, choline in the cell wall, and amounts of CBPs PspA, CbpA and LytA. The sensor regulators appear to respond to small peptides released by the bacteria in a cell-density-dependent fashion. These multiple regulatory circuits allow the pneumococcus to progress through cell-density-dependent changes in physiology: transformation at low density, adherence and

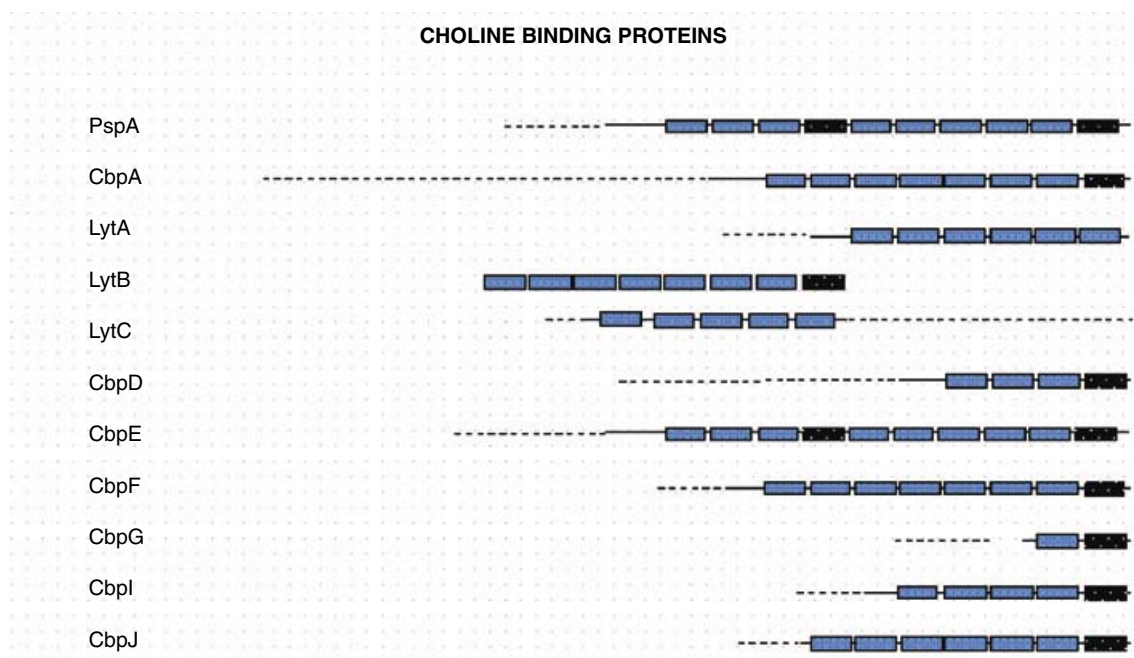**CHOLINE BINDING PROTEINS**

Fig. 11.   The choline binding protein (CBPs) family. Twelve cbps have been described. Each contains a choline binding domain which is composed of a different number of repeat units (blue box). The functional portion of each cbp differs.

invasion in mid-logarithmic phase and autolysis in stationary phase.

The CBP family includes such important determinants as PspA (a well-defined protective antigen), LytA, B, and C (three autolysins), and CbpA (an adhesin). The protective antigen (PspA) is a 65 kD protein with 10 choline-binding repeats. The N-terminus is highly charged, a-helical and both structurally and antigenically variable (McDaniel et al., 1992). PspA appears to inhibit complement-mediated opsonization of pneumococci, and mutants lacking PspA have reduced virulence (Tu et al., 1999). Antibodies against PspA confer passive protection in mice challenged with pneumococcal strains of various capsular serotypes.

The autolytic enzyme (LytA) is responsible for pneumococcal lysis in stationary phase as well as in the presence of antibiotics (Tomasz et al., 1970). This protein has two functional domains: a C-terminal domain with six choline-binding repeats that anchor the protein on the cell wall, and an N-terminal domain that provides amidase catalytic function. Autolysin LytB is a glucosaminidase involved in cell separation and LytC exhibits lysozyme-like activity (Garcia et al., 1999a; Garcia et al., 1999b). Autolysin-negative mutants have not been studied extensively for virulence in vivo.

A major pneumococcal adhesin (CbpA) has eight choline-binding repeats. The N-terminus

(amino acids 1 to 433) consists of 2 repeats, each containing 3 a-helices. A CbpA-deficient mutant is not only defective in colonization of the nasopharynx in an infant rat model but also fails to bind to various human cells in vitro and can not cross a blood–brain barrier in vitro (Rosenow et al., 1997; Ring et al., 1998). The molecule also has been reputed to bind to secretory IgA and the third component of complement (Hammerschmidt et al., 1997; Smith and Hostetter, 1998).

Adherence and Invasion

Pneumococci are low-efficiency invaders in that a maximum of 0.2% of an inoculum enters cells (Cundell et al., 1995). This figure is tenfold lower than other streptococci. Clinical isolates exhibit a wide variability in invasive capacity, at least in part due to effects of the capsule. Invasion of host cells is believed to be a multistage process initiated by adherence. For resting nasopharyngeal cells and type II pneumocytes, simple, primary attachment of pneumococci is mediated by as yet unidentified bacterial lectins. Inhibition studies with soluble sugars suggest the bacteria recognize host cell glycoconjugates bearing N-acetyl-D-galactosamine linked either β1-3 or β1-4 to galactose (Cundell and Tuomanen, 1994). These interactions are distinct from those lead-

ing to invasion because a) they are not dependent on quorum-sensing events, b) there is no difference between opaque and transparent phase variants, and c) they do not directly result in symptomatic disease.

In a minority of individuals, pneumococci are cleared from the nasopharynx in a few weeks' time. However, for a minority of individuals, the bacterial invasion progresses from the nasopharynx into blood, middle ear or the respiratory tract. Progression to invasive disease is facilitated by the activation of host cells providing new opportunities for pneumococci to tether to target cells. CbpA is the major adhesin for activated cells. In addition, choline itself serves as a direct ligand for binding to the PAF receptor (Cundell et al., 1995). Pneumococci are low-efficiency invaders, with only 0.2% of the adherent population reaching an intracellular vacuole. Transcytosis is restricted to the transparent pneumococcal phenotype and is completely absent in mutants lacking the adhesin, CbpA. Once intracellular, the number of viable pneumococci decreases steadily as a result both of intracellular death as well as exit from the cell either by transcytosis through the cell or recycling back to the original port of entry. This recycling is well described for the PAF receptor. The pneumococcal-bearing vesicle is driven across the cell by an as yet unknown intracellular signaling mechanism activated by PAF receptors. The in vivo relevance of these in vitro observations has been suggested by the ability of a PAF-receptor antagonist, administered at the time of intratracheal pneumococcal challenge, to greatly attenuate the bacterial load in the lung and prevent bacteremia (Idanpaan-Heikkila et al., 1997).

Threshold for Disease

In healthy tissues, it requires challenge with ~100,000 bacteria/ml to trigger an inflammatory response (Tuomanen et al., 1985). In contrast, the pneumococcus becomes invasive and inflammation ensues with as few as 10 bacteria if a preceding proinflammatory signal is supplied. This signal is a cytokine in experimental systems or an antecedent viral infection in clinical situations. The inflammatory response can cause considerable tissue damage. In addition, as pneumococci begin to lyse in response to host defense molecules and antimicrobial agents, they release cell wall, pneumolysin and other components that lead to greater inflammation and cytotoxic effects. Pneumolysin and hydrogen peroxide not only kill cells, but also strongly induce production of nitric oxide (Braun, submitted). This potent vasoactive mediator is

a major agent operative in septic shock. In addition to direct damage by cytotoxins and to bystander effects during an exuberant host response, human cells can undergo apoptosis in response to pneumococci. This is especially evident during meningitis where neurons of the hippocampus are killed by this mechanism (Zysk et al., 1996). New studies suggest that significant protection from neuronal injury can be achieved by inhibition of apoptosis by caspase inhibitors (Braun et al., 1999). Downmodulation of the host inflammatory response during the early phases of antibiotic therapy has proven successful as an adjunctive therapy to reduce sequelae in meningitis (Tuomanen et al., 1987; Lebel et al., 1988).

Schema of Pathogenesis: Pneumonia, Bacteremia and Meningitis

Transparent pneumococci adhere tightly to the nasopharyngeal epithelium by multiple mechanisms that, for most individuals, appear to result in a benign course generating type-specific immunity. For some people, however, progression into the lungs or middle ear occurs. Passage of pneumococci up the eustachian tube is accompanied by bacterial induced changes in the surface ligands of the epithelial cell, particularly by neuraminidase (Linder et al., 1994). Inflammation in the middle ear is driven by cell wall (Carlsen et al., 1992), and pneumolysin inflicts major cytotoxicity on ciliated cells of the cochlea (Comis et al., 1993). Upon reaching the lower respiratory tract by aerosol, pneumococci bypass ciliated upper respiratory epithelial cells unless there is damage to the epithelium. Rather, they progress to the alveolus and associate with the type II alveolar cell, the cell that produces choline-containing surfactant (Tuomanen et al., 1995). Although transparent colony variants seem to have a clear advantage in their initial encounter with nasopharyngeal epithelial cells, they appear to have no advantage in the naive lung. The high level of adherence of transparent forms is only apparent upon activation of the lung cells, for instance, by intercurrent viral infection or other cytokine-producing event. These activated cells then display new receptors that facilitate pneumococcal invasion, a process predisposing to bacteremia.

The ligands by which pneumococci bind to activated human cells include choline on the cell wall teichoic acid that can serve as a direct ligand to the PAF receptor, and the choline-binding protein, CbpA. Transparent variants of pneumococci adhere more efficiently to the activated pulmonary epithelium because they display both more choline to interact directly with the upreg-

ulated PAF receptor and more CbpA to interact with pulmonary epithelial surface carbohydrates. Once bound to the PAF receptor, the pneumococcus enters a vacuole in a PAF receptor-dependent endocytic process and the vacuole moves across the cell expelling the bacteria on the ablumenal surface. In vitro, pneumococci will adhere to and traverse an endothelial barrier over approximately 4 hours.

If high grade bacteremia is established, the risk of meningitis increases. Pneumococci can adhere specifically to cerebral capillaries using the same pairings of choline to PAF receptor and CbpA to carbohydrates (Ring et al., 1998). Thus, the bacteria subvert the endocytosis/recycling pathway of the PAF receptor for cellular transmigration. Once in the CSF, a variety of pneumococcal components, particularly cell wall, incite the inflammatory response (Tuomanen et al., 1985). Cytochemical and pathophysiologic differences occur in experimental meningitis caused by different strains of *S. pneumoniae* depending on their ability to release cell wall components. In vitro and in vivo experiments have demonstrated rapid killing and lysis of bacteria when penicillin is administered during an experimental infection. Based on the inflammatory potential of the cell wall and its rapid release during antibiotic therapy, a rationale for downregulation of inflammation in the early therapeutic time window has arisen. Dexamethasone shows favorable benefit to outcome of disease when given with the first several doses of antibiotic (Lebel et al., 1988), presumably by preventing increases in inflammation and consequent neuronal loss. Other avenues that could accomplish similar results include blocking leukocyte accumulation in the cerebrospinal fluid, inhibiting cytokine activity, decreasing production of oxygen and nitrogen radicals and nitric oxide, or preventing activation of caspases that promote apoptotic cell death (Braun et al., 1999; Quagliarello and Scheld, 1997). The ability to improve survival by modulating the molecular course of events between pneumococcus and host inflammation is an area of rapid current progress.

"Increased power to destroy the diplococcus is not associated necessarily with an equally increased power to resist the toxic effects of the intracellular poison. This fact must have an important bearing upon a specific therapy of diplococcus meningitis" (B. White, 1938).

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CHAPTER 1.2.5

The Genus Enterococcus: Taxonomy

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Introduction

The genus *Enterococcus* contains bacterial spe-
cies associated with animals and plants. Only
species from humans and domestic animals have
been studied in some detail. Limited information
is available on plant-associated species and this
has been mainly derived from the study of strains
transiently associated with humans or animals.

   The enterococci are most often considered as
components of the intestinal flora of humans and
animals acting as opportunistic pathogens in dif-
ferent extra-intestinal compartments of the body.
They have received considerable attention in
medical bacteriology because of their increasing
role in hospital-acquired (nosocomial) infec-
tions. An important factor contributing to this
phenomenon undoubtedly has been their natural
(intrinsic) and acquired resistance to frequently
used antibiotics. Numerous studies have been
devoted in recent years to this topic. Genetic
studies (not treated in the present contribution),
except those undertaken for taxonomic pur-
poses, have largely concerned plasmids and
transposons in connection with antibiotic resis-
tance, and two genetic systems that have been
described in *Enterococcus faecalis*: conjugative
plasmids and sex pheromone plasmids.

Phylogeny

Relation to Other Genera

The enterococci have been separated from the
streptococci, first based on DNA-DNA and
DNA-rRNA hybridization studies (Schleifer and
Kilpper-Bälz, 1984; Schleifer et al., 1985; Schlei-
fer and Kilpper-Bälz, 1987). This separation was
confirmed by 16S rRNA sequence analysis (Lud-
wig et al., 1985) which showed that the entero-
cocci also differed from the lactococci and
certain other Gram-positive cocci. The entero-
cocci belong to the Firmicutes with low G+C
content, the so-called clostridial branch.
Phylogenetically the closest relative of the
enterococci, but well separated from the latter,

is the genus *Vagococcus* and next *Carnobacte-
rium*, *Tetragenococcus*, *Aerococcus*, *Alloiococ-
cus*, *Dolosigranulum*, *Facklamia*, *Globicatella*
and *Abiotrophia* (Collins et al., 1997). The strep-
tococci and the lactococci to which the entero-
cocci have been linked in the past, are more
distantly related, as are the lactobacilli.

Species Groups

16S rRNA Reverse Transcriptase Sequence
Analysis.
   Within the genus certain groups of species
(Table 1) have been shown by 16S rRNA reverse
trancriptase sequence analysis to be more closely
related to each other than to others (Williams et
al., 1991). *Enterococcus faecalis* forms a distinct
lineage, as do *E. saccharolyticus*, *E. sulfureus* and
E. dispar. The intraspecies group distances
between *E. cecorum* and *E. columbae* are larger
than the distances seen within other species
groups. Patel et al. (1998) produced by the same
technique a distance matrix tree that was nearly
identical except for the fact that *E. sulfureus* and
E. saccharolyticus appeared to form still another
group with its two distantly related members.

Relation to Phenotypic Characteristics

Most interestingly, these "species groups" show
a fairly large number of phenotypic characteris-
tics which are typically shared by all members of
a given group (Devriese et al., 1993b). These
phylogenetic groups are therefore useful natural
groups whose common characteristics can be
used for identification. It is far easier to differen-
tiate the various species groups from each other
than the species within the groups. Moreover, a
number of important characteristics are common
and unique to certain species groups. For exam-
ple the *E. cecorum* group is carboxyphilic and
does not exhibit the unusual resistance to drying
commonly attributed to the enterococci. All
strains of the *E. gallinarum* group possess the
vanC-gene cluster conferring low-level resis-
tance to glycopeptide antibiotics such as
vancomycin.

Table 1. Phylogenetic enterococcal species groups as determined by reverse transcriptase DNA sequence analysis of 16S rRNA.

Species group	Species
E. faecium group	*E. faecium*
	E. durans
	E. hirae
	E. mundtii
E. avium group	*E. avium*
	E. malodoratus
	E. raffinosus
	E. pseudoavium
E. gallinarum group	*E. gallinarum*
	E. casseliflavus
E. cecorum group	*E. cercorum*
	E. columbae

From Williams et al. (1991).

Table 2. *Enterococcus* species.

Species	Described by
E. faecalis	Schleifer and Kilpper-Bälz, 1984
E. faecium	Schleifer and Kilpper-Bälz, 1984
E. durans	Collins et al., 1984
E. gallinarum	Collins et al., 1984
E. casseliflavus	Collins et al., 1984
E. avium	Collins et al., 1984
E. malodoratus	Collins et al., 1984
E. hirae	Collins et al., 1986
E. mundtii	Collins et al., 1986
E. pseudoavium	Collins et al., 1989
E. raffinosus	Collins et al., 1989
E. cecorum	Williams et al., 1989
E. columbae	Devriese et al., 1990
E. saccharolyticus	Rodrigues and Collins, 1990
E. dispar	Collins et al., 1991
E. sulfureus	Martinez and Collins, 1991
E. asini	de Vaux et al., 1998

Formal infraspecies divisions have not been made in the genus, though some ecovar-related variability has become apparent in *E. faecium*. These ecovars pertain to biochemical reaction types (biotypes), and more convincingly, genotypes associated with certain animal host species (Devriese et al., 1987; Quednau et al., 1999).

Other Techniques

A phylogenetic tree derived from sequences of internal fragments of structural D-alanine: d-alanine ligase genes and alignments of deduced amino acid sequences was found to be largely superposable on that derived from 16S rRNA sequences (Evers et al., 1996). Similar attempts to determine subdivisions with other techniques proved less satisfactory. Polymerase chain reaction (PCR) amplification of the intergenic spacer (ITS-PCR) between the 16S and 23S rRNA, as determined by Tyrrell et al. (1997), recognized the *E. avium* group as well as *E. hirae* with *E. durans*, but failed to separate *E. faecalis* from *E. faecium* or *E. gallinarum*. Broad-range PCR (BR-PCR) amplification (Monstein et al., 1998) of 16S rDNA fragments including variable regions V3, V4 and V9 resulted in 12 different species groups, which partially corresponded to the 16S rRNA species groups determined by reverse transcriptase sequencing of the nearly complete 16S rRNA genome by Williams et al. (1991). Randomly amplified polymorphic DNA (RAPD) analysis using the unweighted pair group method of association (UPGMA) clustering showed good agreement with the 16S rDNA groups and individual species (Monstein et al., 1998).

Taxonomy

Classification Errors and Problems

About twenty species have been allocated to date (August 1999) to the genus *Enterococcus*. However, certain taxonomic and nomenclatural difficulties are apparent. *Enterococcus solitarius* (Collins et al., 1989) has been shown to be more closely related to the genus *Tetragenococcus*

(Collins et al., 1990; Williams et al., 1991). *Enterococcus seriolicida* (Kusuda et al., 1991) is identical to *Lactococcus garvieae* and has to be reclassified as such (Teixeira et al., 1996).

Another problem concerns *Enterococcus flavescens* (Pompei et al., 1991). This species appears to be identical to *Enterococcus casseliflavus*, which has nomenclatural priority. Neither protein analysis nor PCR-based typing was able to differentiate between strains allocated to either one of the two species (Descheemaeker et al., 1997). Their ligase genes showed high levels of similarity (Navarro and Courvalin, 1994; Dutka-Malen et al., 1995).

Melissococcus pluton (Bailey and Collins, 1982), the etiological agent of European foulbrood disease of honey bees, is phylogenetically closely related to the genus *Enterococcus* (Cai and Collins, 1994; de Vaux et al., 1998). Despite some doubts, the genus *Melissococcus* has been retained as a separate genus for nomenclatural convenience and because of its branch point at the periphery of the the *Enterococcus* cluster.

Current Species

These observations taken into consideration, 17 *Enterococcus* species are to be retained (Table 2).

Formal infraspecies divisions have not been made in the genus, though some ecovar-related variability has become apparent in *E. faecium*. These ecovars pertain to biochemical reaction

types (biotypes), and more convincingly, genotypes associated with certain animal host species (Devriese et al., 1987; Quednau et al., 1999).

Habitat

Intestinal

The best known, though not the only habitat of the enterococci, is the gut of mammals and birds. They may be significant components of other animal groups as well. Apart from some sporadic observations, few data are available on this topic (see, for example, Benno et al., 1992 on *E. faecalis* in the Japanese tree frog, *Hlya japonica*). Most enterococcal species known to date are typically associated with the intestines of humans and domestic animals, and when found outside the gut, they are interpreted as indicators of fecal pollution. Others, notably the plant-associated yellow-pigmented *E. casseliflavus* and *E. mundtii* (Collins et al., 1986; Vaughn et al., 1979) may occur transiently in the intestines.

Host Species Variation

Certain host-specific variations in the occurrence of different species in different animal hosts are known to exist. In humans as well as in many other animal species, *E. faecalis* and *E. faecium* are most frequently found. The first is more common and usually occurs in larger numbers than the second (see review in Murray, 1990). *E. cecorum* is a prominent member of the enterococcal flora of poultry and pigs (Devriese et al., 1991a; Devriese et al., 1994), whereas *E. columbae* is the dominant component of the gut flora of pigeons. *E. hirae* is a frequent inhabitant of the porcine gut and may occur in poultry, cattle, dogs and cats (Devriese et al., 1987). *E. durans* has been isolated from humans, chickens and calves. Despite its name, *E. avium* is rarely isolated from the intestines of birds, and *E. gallinarum*, similarly, is not an important member of the flora of chickens. The habitat of the members of the *E. avium* species group (*E. avium*, *E. malodoratus*, *E. raffinosus* and *E. pseudoavium*) is largely unknown. *E. malodoratus* is often found in the tonsils of cats (Devriese et al., 1992a; author's correction in this reference: *E. raffinosus* is to replaced by *E. malodoratus*).

Age Variation

In certain hosts, variations in the enterococcal flora according to age have been documented. Enterococci are among the dominant flora of the intestine in the very first days of life in many animals, but they decline to markedly lower lev-els at 2 to 3 weeks of life (Smith and Crabb, 1965). In ruminants they are frequent in the pre-ruminating period but they decline to very low levels later on (Devriese et al., 1992). Age-dependent variations in species distribution have been observed in the enterococcal flora of chickens: *E. faecalis* and *E. faecium* prevail during the first days of life. Later on *E. faecalis* starts to decrease first, followed by *E. faecium*, to be replaced by *E. cecorum* (Devriese et al., 1991b). Also in humans, *E. faecalis* largely outnumbers the other species in infants less than 1 week of age (Noble, 1999).

Variation in Different Compartments

The enterococcal flora may differ in different compartments of the intestine as has been documented in chickens (Devriese et al., 1991a): *E. durans* and *E. hirae* were part of the small intestinal flora of 3- to 4-week-old chicks but were not detected in the crop and the caeca of the same animals.

Variation Due to Feeding

A well-known though somewhat special example of the influence food ingestion may have is the low enterococcal content of feces from breast-fed infants (mean count per gram in 4–7 week-olds: 6.3 $\log_{10}$) compared with formula-fed infants (9.6 $\log_{10}$; Stark and Lee, 1982). Other food-dependent variations are much less clear.

Other Body Sites

Enterococci may also occur in the throat and in the vagina of humans, usually less than 20% of individuals being positive. Most isolates are *E. faecalis*, though in certain hospital settings *E. faecium* may outnumber *E. faecalis* (McGowan and MacFarlane, 1983).

Association with Plants and Invertebrates

Certain species are known to be typically plant-associated. This is notably the case with the yellow-pigmented *E. mundtii* and *E. casseliflavus* (Martin and Mundt, 1972). However, *E. faecium* and *E. faecalis* are also frequently isolated from this source (Ulrich and Muller, 1998). *E. casseliflavus* was the predominating species in forest industry wastewater in Finland. Enterococcal-like strains from pristine waters could not be identified except for some rare *E. faecalis* isolates (Niemi et al., 1993). In moderate climates, the enterococci disappear from the plant world during the winter, reappearing during the spring and becoming more and more frequent as the plants grow and flourish. It has been speculated

that insects play an important role in this seasonal variation (Martin and Mundt, 1972). Undetermined *Enterococcus* strains have been shown to play a role in the gut metabolism of wood-feeding termites (Tholen et al., 1997).

Isolation

Enterococci are usually isolated from pathological specimens on nonselective blood agars or on blood agar supplemented with colistin (polymyxin) and nalidixic acid or oxolonic acid active against mainly Gram-negative bacteria. These are most useful additions. Experienced people may be able to recognize *E. faecalis* colonies on blood agar relying on their relatively large nonhemolytic or β-hemolytic colonies. Most *E. faecium* group strains produce α-hemolytic colonies. This approach yields only presumptive identifications, of course.

Many different selective media have been devised for the enterococci but none has proved specific (Reuter, 1992). Sodium azide is the main selective component in most of them. In certain formulations bile salts or antibiotics (such as neomycin or gentamicin) with poor anti-enterococcal activity are added, and aesculin or tri-tetrazolium figure as indicator substances. Higher temperatures (42 to 45C°) may be applied as well to improve enterococcal selectivity (Niemi and Ahtiainen, 1995). One of the most used media is Slanetz and Bartley agar (also named M-*Enterococcus* agar). The visual quality and selective capacity of this medium may vary depending on the heat exposure of tetrazolium during preparation, though this affects the yield of intestinal streptococci such as *S. bovis* and *S. gallolyticus* much more than the enterococci. Usually, *E. faecalis* colonies can be recognized on this medium, which confers to this medium an advantage over others.

It should be known that not all enterococcal species grow on enterococcal selective media (Devriese et al., 1993b).

Identification

Genotypic Methods

Several genotypic methods have been evaluated for their ability to identify enterococcal strains to species level. Full sequencing of the 16S-rRNA gene was done by Williams et al. (1991) and by Patel et al. (1998). On the basis of these sequences, a phylogenetic tree was constructed from which species groups can be distinguished. Homology values within the genus *Enterococcus* ranged from 93.7% to 99.8% for a 1,452-nucleotide region (Williams et al., 1991). Because this is a costly and time-consuming method, several other tools have been studied for the ability of identification.

A species-specific PCR assay has been developed (Dutka et al., 1995). This multiplex PCR assay makes use of four primer pairs. Two primer pairs are directed to the genes encoding D-alanine:D-alanine ligases (*ddl* genes), one pair being complementary to *ddlE. faecium*, another to *ddlE. faecalis*. The *vanC-1* and *vanC-2* genes are specific for *E. gallinarum* and *E. casseliflavus*, respectively. These genes encode for intrinsic vancomycin resistance.

Tyrrell et al. (1997) used intergenic ribosomal PCR, which amplifies the noncoding region between the 16S and 23S rRNA genes, to discriminate enterococcal strains to species level. Profiles of several species, such as *E. avium*, *E. raffinosus*, *E. malodoratus* and *E. pseudoavium* and also *E. faecalis* and some *E. hirae* strains, were highly similar. Differentiation was made possible by digestion of the amplification products with *Sau3A*, except between *E. avium* and *E. pseudoavium*.

Descheemaeker et al. (1997) and Quednau et al. (1998) investigated the usefulness of RAPD in the identification of enterococci. The use of primer D11344 resulted in different amplification patterns for the species *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, *E. gallinarum* and *E. casseliflavus* (Descheemaeker et al., 1997). Quednau et al. (1998) could visually distinguish all clinically relevant species on the basis of their fingerprint without the need of computer-based analysis. In both studies, *E. flavescens* strains showed the same pattern as *E. casseliflavus*, thus confirming that the former name is to be disregarded.

In the multiplex PCR of Dutka-Malen et al. (1995), speciation is limited to *E. faecium*, *E. faecalis*, *E. gallinarum* and *E. casseliflavus*. The RAPD and other assays may be made applicable to any desired species, provided that the discriminatory capacity and interlaboratory reproducibility of the method is satisfactory.

Phenotypic Methods

GENUS IDENTIFICATION The classical species *E. faecalis* and *E. faecium*, as well as the species forming a species group with the latter, have a number of characteristics in common which separate them to some extent from the other Gram-positive, catalase-negative, facultatively anaerobic cocci: ability to grow in 6.5% NaCl broth, at pH 9.6, at 10°C and at 45°C; presence of group D antigen. These characteristics which traditionally are attributed to the enterococci are far from common to the other enterococcal species. Espe-

Table 3. Characteristics common to all or nearly all enterococci.

Characteristic	Result
VP	+ (negative in *E. saccharolyticus*)
β-Glucosidase	+
β-Glucuronidase	− (positive in most *E. cecorum* strains)
Urease	−
Resistance to 40% (v/v) bile	+
Aesculin hydrolysis	+
Acid from	
N-acetylglucosamine	+
Amygdalin	+
D-Arabinose	−
Arbutin	+
Cellobiose	+
Erythritol	−
D-Fructose	+
Galactose	+
β-Gentiobiose	+
Glucose	+
Glycogen	− (positive in some *E. gallinarum*, *E. cecorum* and *E. columbae* strains)
Inositol	− (delayed positive in *E. raffinosus*)
D-Fucose	−
L-Fucose	−
Lactose	+ (negative in certain *E. faecalis* strains)
Maltose	+
D-Mannose	+
Methyl-β-D-glucopyranoside	+ (not reported in some of the newer species)
α-Methyl-D-xyloside	−
Pullulan	− (not reported in some of the newer species)
Ribose	+ (negative in *E. asini* and in some *E. casseliflavus* strains)
Salicin	+
Trehalose	+ (negative in some *E. faecalis* strains)
L-Xylose	−

VP = Voges Proskauer test.

cially the newer species are often negative in one or more of these tests.

A fairly large number of other characteristics are found in nearly all enterococci (Table 3) but, with certain useful though not absolute exceptions, they are not specific for the genus. The VP (Voges-Proskauer or acetoin reaction) and acid production from ribose have a high differential value especially with regard to the streptococci. Only *S. agalactiae*, *S. uberis* and the β-hemolytic *S. porcinus* react positive in both tests as do all enterococci, except *E. saccharolyticus* (VP−) and *E. asini* and some *E. casseliflavus* strains (ribose−).

Although no single phenotypic test or combination of tests is able to characterize the genus *Enterococcus* adequately, certain practical approaches can be used. Enterococcal-like colonies growing to "normal" colony size on media containing 0.04% sodium azide selective for enterococci and able to grow in 6.5% NaCl broth are most probably enterococci. In case of doubt, VP and/or ribose testing can be added. Typically, only streptococci of the *Streptococcus bovis* spe-

cies group show colony characteristics similar to those of the enterococci on these media. These streptococci are always ribose-negative and they do not grow in 6.5% NaCl broths.

This procedure is only valid when the "classical" enterococci are looked for exclusively, and when the newer species can be disregarded.

SPECIES GROUPS The enterococcal species groups differ from each other in a number of characters which are useful to confirm identifications (Table 3). A more extensive version of this table has been published by Devriese et al. (Devriese et al., 1993b).

SPECIES Identifications of species within a species group are more difficult to make, and errors are more likely to occur within groups than between groups. Further details are provided by Devriese et al. (1993b). In routine diagnostic bacteriology, usually presumptive identifications based on growth characteristics will be confirmed, and phenotypic identifications will be made by using identification galleries or applying

Table 4. Identification of species groups.

Test	E. faecalis	Faecium group	Avium group	Gallinarum group	Cecorum group
Motility	−	−	−	+	D
Capnophilic growth	−	−	−	−	+
Group D antigen	+	D+	D	+	−
APPA	−	−	+	−	−
PYRA	+	+	+	+	−
Alkaline phosphatase	−	−	−	−	D+
α-Galactosidase	−	D	D	+	+
Arginine dihydrolase	+	+	−	+	−
Acid from					
Adonitol	−	−	D+	−	−
L-Arabinose	−	D	D+	+	D
D-Arabitol	−	−	+	−	D
α-Methyl-D-glucopyranoside	−	−	+	+	D
D-Raffinose	−	D	D+	D	+
L-Sorbose	−	−	+	−	−

short identification schemes such as the one produced by Facklam and Collins (1989). These procedures are reliable with nearly all *E. faecalis* and most *E. faecium* strains, but less trustworthy with the others.

Certain of the tests differentiating between groups indicated in Table 3 are particularly useful to confirm identifications of the less well-known species. An example of such use is the acidification of methyl-α-D-glucopyranoside, which differentiates *E. gallinarum* and *E. casseliflavus* (the *E. gallinarum* group) from *E. faecalis* as well as from *E. faecium* and related organisms (Devriese et al., 1996).

Preservation

Enterococcus strains are notoriously resistant to adverse environmental conditions such as drying, which makes preservation easy. This is to be nuanced, however, in that certain species, notably *E. cecorum*, are not as resistant as others. The classical enterococci can be preserved for many years at −20°C in cryopreservative media (lyophylization media). Caution is warranted when the less well-known species are involved. Preservation at −70°C or lyophilization is recommended for such strains as well as for strains whose characteristics are to remain as intact as possible for certain applications and research purposes.

Epidemiology

The epidemiology of enterococci has mainly been focused on the species *E. faecium* and *E. faecalis*. The epidemiology of glycopeptide-resistant *E. faecium* has been investigated most

intensely. These studies concerned hospital epidemiology and the possible spread of resistant strains from animals to humans.

Tools

The epidemiology of enterococci has been investigated with different molecular techniques, and depending on the method used, different and frequently opposite conclusions have been drawn. The highest discriminative power was obtained using PFGE (pulsed-field gel electrophoresis). This technique can clearly divide the enterococcal species into diverse clones. Ribotyping has been shown to be less suitable in typing *E. faecalis* strains (Gordillo et al., 1993). Restriction endonuclease analysis (REA) is discriminative in *E. faecium*, and may yield valuable results comparable to those obtained with PFGE. No single ideal method can be used without clinical epidemiological investigation, but any of these techniques is helpful (Savor et al., 1998). Biochemical characteristics do not allow distinction of enterococcal strains from different host species. However, raffinose-positive *E. faecium* strains are found solely among poultry strains (Devriese et al., 1987).

Transfer Between Host Species

Studies on the transfer of enterococci between host species have mainly focused on the transfer of vancomycin-resistant *E. faecium* from animals to humans. Ribotyping did not differentiate between vancomycin-resistant *E. faecium* strains from different host species (Bates et al., 1994). By using PFGE, a high genetic variability was found among the strains (Klare et al., 1995) and transfer between hosts appeared to be uncommon (Descheemaeker et al., 1999; Klare

et al., 1995; vandenBraak et al., 1998). However, on some occasions similar strains were found in both animals and humans (Descheemaeker et al., 1999; Jensen, 1998; Stobberingh et al., 1999).

Using REA of total DNA on *E. faecium* strains, a clear division could be made between strains from different animal hosts and humans (Quednau et al., 1999). This was the first firm indication of the host specificity of enterococci.

Nevertheless, transient in vivo colonization of the human gut by animal strains is possible when high doses are administered. Enterococci do not seem to survive in a different host for a prolonged period (Berchieri, 1999).

Hospital Epidemiology

Traditionally, enterococcal infections have been considered to be endogenous, arising from the patient's own flora. More recently, however, they have been shown to spread from patient to patient and from hospital to hospital. Health care personnel and inanimate objects may be responsible for transmission (Korten and Murray, 1993). In hospitals, the clonal spread of resistant strains, mainly vancomycin-resistant *E. faecium* strains, has been investigated most often by using PFGE. It has been demonstrated that some multiple resistant strains could spread within a hospital and between hospitals, even over a period of 6 years. However, equally as many outbreaks were polyclonal (McDonald et al., 1997).

Disease

Enterococci used to be known only as causes of endocarditis and rare cases of meningitis. This picture has changed considerably in the last decade: these bacteria have become one of the leading causes of nosocomial (hospital-acquired) bacteremia, and of surgical and urinary tract infections.

Infections Caused by Enterococci

IN HUMANS The most frequent form of enterococcal disease, urinary tract infection, is most often caused by instrumentation or structural abnormalities of the urinary tract. Prior antibiotic therapy is another risk factor. Use of antibiotics especially those lacking effective antienterococcal activity such as cephalosporins, fluoroquinolones, polymyxins, macrolides, lincosamides and potentiated sulfonamides, is an important predisposing factor. Acquired resistance against other agents may aggravate the situation (Gray and Pedler, 1992).

Intra-abdominal and pelvic infections are often polymicrobial, but the enterococci are important components of the infecting flora. They are involved in peritonitis associated with chronic ambulatory peritoneal dialysis, spontaneous peritonitis in cirrhotic or nephrotic patients, as well as in acute salpingitis, pelvic abscesses and other forms of peripartum pathology.

Enterococcal bacteremia is associated with endocarditis in a minority of cases, other conditions such as urinary tract infection being much more frequent. Intraurinary or intravascular catheters are often involved. Polymicrobic bacteremia is very common, and mortality is generally high, most probably because of severe underlying disease and complicating factors. In countries such as the United States, enterococci rank among the most common causes of bacteremia, but in others they may be much less frequent (Pfaller et al., 1999). Endocarditis due to enterococci is seen most frequently in elderly males, often suffering urinary tract infection or undergoing invasive tests involving instrumentation. Rarer disease conditions include neonatal infections and infection of the central nervous system. Most cases represent complications of underlying disease.

IN ANIMALS Animals are not hospitalized except for some pets, and even these are much less frequently hospitalized than humans. Debilitated and aged animal patients are less often treated. For this reason the typical enterococcal pathology seen in humans is virtually unknown in animals.

Nevertheless, certain pathological conditions associated with enterococci have been documented. Most often, birds appear to be involved and *E. hirae* strains have been implicated as causes of septicemia and focal brain necrosis (Devriese et al., 1991b). Other *E. hirae*-like or *E. durans*-like strains have been shown to adhere to the intestinal villi of sucklings of several mammalian species, and may cause relatively mild enteritis (Cheon and Chae, 1996; Dooley, 1998).

PATHOGENICITY Among the enterococci *E. faecalis* is the species most frequently associated with disease in humans. Although *E. faecium* strains have become resistant to antibiotics more often than *E. faecalis* strains, the relative importance of these species does not appear to change dramatically (Huycke et al., 1998). This suggests that *E. faecalis* is more virulent or that *E. faecalis* strains more often possess virulence factors. These include cytolysin, pheromone-responsive plasmid transfer with production of aggregation substance, extracellular superoxide production, and a surface protein (designated as Esp). Their

pathogenic role is still unclear for the greater part.

CYTOLYSIN Cytolysin production has been shown to be clearly associated with *E. faecalis* strains isolated from pathological conditions (Ike et al., 1987). Enterococcal cytolysin causes lysis of different target membranes including those of erythrocytes, resulting in hemolysis on some types of blood agar. It has been shown to contribute to the severity of several experimental infections and is associated with increased risk of sudden death from nosocomial bacteremia reviewed in (Huycke et al., 1998). The cytolysin is expressed and processed through a complex maturation pathway (Booth et al., 1996).

SEX PHEROMONE AND AGGREGATION SUBSTANCE Adherence is important in the pathogenesis of *E. faecalis* urinary tract infection and endocarditis (Guzman et al., 1989). One possible mechanism underlying adherence is mediated by small peptides seven to eight amino acids in length called pheromones. Sex-pheromone plasmid-carrying *E. faecalis* donor cells are stimulated by the excretion of pheromones by plasmid-free potential recipient cells, to synthesize a corresponding adhesive protein, called aggregation substance. This results in a tight aggregation of both types of cells, thus making the conjugative transfer of sex-pheromone plasmid possible. This unique system is remarkably regulated and versatile: inhibitor peptides are excreted by donor cells which neutralize the effects of the corresponding sex pheromones, and donor cells may produce sex pheromones not related to the sex-pheromone plasmid they harbor. The prototype of this class of genetic elements is Tn*916*.

The N-terminal part of the adhesin is responsible for the clumping effects, with a region between amino acid 525 and amino acid 617 playing a dominant role (Muscholl, 1998). This aggregation substance mediates also adhesion to eukaryotic cells such as cultured renal tubular cells (Kreft et al., 1992), and it has been shown to enhance pathogenicity in animal models of *E. faecalis* endocarditis (Schlievert et al., 1998), though in other infection models it appears to play a less determinative role (Dupont et al., 1998). The role of plasmid-encoded aggregation substance in the transition from bacteremia to endocarditis is still a matter of debate (Berti et al., 1998).

SUPEROXIDE PRODUCTION Nearly all *E. faecalis* and very few *E. faecium* strains produce substantial amounts of extracellular superoxide, and production is higher in strains from septicemia than in strains from carriers. The role of this sub-stance in the pathogenicity of *E. faecalis* is still unknown (Huycke et al., 1998).

ESP Esp is a cell wall-associated protein of unusual repeating structure (Shankar et al., 1999) which bears global organizational similar-ity to the Rib and C-α- proteins of group B streptococci. It has been detected in infection-derived *E. faecalis* strains but not in other enterococcal species. Its role in disease is as yet unsure.

ANTIBIOTIC TREATMENT Most enterococcal infections are treated with single-drug therapy. Ampicillin, penicillin, vancomycin have been used most often. Classically, β-lactams and amino-glycoside antibiotics are combined to treat endocarditis. However, these regimens are inad-equate when strains with high-level aminogly-coside resistance are involved, and facing resistance situations single-drug policies may have to be changed as well.

ANTIBIOTIC RESISTANCE Enterococci are intrinsi-cally resistant to many antibiotics. Certain β-lactams such as penicillin, ampicillin, piperacillin and imipenem show good bacteriostatic activity (Huycke et al., 1998). Combinations of these β-lactam antibiotics with aminoglycosides have been most widely used to achieve bactericidal effects. The glycopeptides vancomycin and teico-planin have been valuable alternatives.

Therapeutic possibilities are hampered by increasing numbers of strains with acquired resistance, especially among *E. faecium*. Resis-tance determinants against all useful antibiotics in the treatment of enterococcal infections have been described, even against the newest agents, which as yet have not been used extensively. Important regional differences in resistance prevalence have been noted (Pfaller et al., 1999).

Resistance against β-lactam antibiotics is mostly due to alterations in the penicillin-binding proteins of the strains. However, it is not clear whether the prevalence of strains with higher resistance levels is increasing or is simply due to selection or identification of resistant strains that were already present naturally (Moellering, 1991). β-lactamase-mediated resis-tance has been reported only in some *E. faecalis* strains isolated in the United States. This resis-tance gene resembles a staphylococcal gene and supposedly the gene has recently been trans-ferred from this genus to the enterococci (Rice et al., 1991).

Only high level resistance against aminogly-cosides is of importance because it affects the β-lactam-aminoglycoside synergy. This type of resistance is mediated by aminoglycoside-modifying enzymes, inactivating these antibiot-ics. The resistance genes are mostly located on

plasmids. Also these genes are identical to the staphylococcal aminoglycoside resistance determinants (Ounissi et al., 1990).

Resistance against glycopeptides including vancomycin has been reported for the first time in 1989 in Europe. Ever since, the number of reports on resistant strains has increased. Especially United States hospitals are coping with increased resistance, in contrast to Europe where infections with GRE (glycopeptide-resistant enterococci) are still uncommon (Schouten et al., 1999).

Animal strains investigated in the United States showed few resistances to therapeutic antibiotics (Thal et al., 1995). Resistance against most antibacterial growth promoters used in animal feed has been demonstrated in enterococci, especially *Enterococcus faecium* isolated from farm animals, pets and foods (Butaye et al., 1999b). Certain of these are potentially important because of their cross-resistance with therapeutically used drugs. The use of avoparcin (a glycopeptide antibiotic) in animal nutrition as a growth-promoting antibiotic has been incriminated as a source of the GRE in Europe. This is not the case in the United States where avoparcin has not been in use, but where high hospital use of vancomycin has resulted in high resistance frequencies. There is still discussion on the impact of glycopeptide resistance among enterococci from animal origin on the resistance of enterococci in humans (Butaye et al., 1999a).

Applications

Enterococci may play a beneficial or a detrimental role in foods. They may cause spoilage or they may contribute to ripening and flavoring processes of certain foods. A special application concerns their use as indicator strains to detect fecal contamination of water, and certain strains are used as additives in feeds or even as therapeutics meant to improve certain intestinal conditions.

Role in Foods

FOOD CONTAMINATION Enterococci are among the most thermotolerant of the nonsporulating bacteria, and the classic species are surprisingly resistant to drying, which makes them prone to cause spoilage in cooked or heated processed meats (Franz et al., 1999). Because of these properties, they can be considered as indicators of sanitary quality of foods. *E. faecalis* is the dominant species in most of these foods.

In marked contrast to this, their presence is highly desirable in a variety of cheeses to achieve certain aromas or other sensory properties. *E. faecium* and *E. faecalis* usually dominate and their numbers are often as high as 10^6 or 10^7 colony forming units/g. In other types of cheese, different lactic acid bacteria predominate, but also in these varieties enterococci represent an important part of the flora of the ripened products (data summarized in Franz et al., 1999). Their proteolytic activity, production of acetaldehyde, acetoin and diacetyl, and possibly also esterase activity on milk fat are considered important for cheese ripening (Centeno et al., 1996; Tsakalidou et al., 1998).

INDICATORS OF FECAL POLLUTION Enterococci are able to survive for long periods on inanimate surfaces even in direct sunlight (Bale et al., 1993). The presence of enterococci in drinking water supplies is monitored mainly because *E. faecalis* and *E. faecium* strains survive much longer than other enteral bacteria in water. Their presence in the absence of *E. coli*, indicates a more distant contamination. Another application concerns the assessment of surface- and recreational water quality (Godfree et al., 1997). The existence of plant-associated species and strains is to be taken into account on certain occasions when fecal pollution is to be verified (Niemi et al., 1993). The detection and enumeration of enterococci in water is carried out by membrane filtration or by enrichment in liquid media (Leclerc et al., 1996).

ADDITIVES The favorable properties of enterococci have led to the inclusion of certain *E. faecalis*, *E. faecium* or *E. durans* in starter cultures of certain types of cheese. They make it possible to achieve desirable properties on a constant basis (Franz et al., 1999).

Another possibly useful characteristic of enterococci concerns their production of bacteriocins active against other enterococci, "*Listeria*" and some "*Clostridium*" species. Bacteriocins produced by *E. faecium* and *E. faecalis* generally belong to class II (Abee et al., 1994; Cintas et al., 1998).

Certain strains of enterococci are in use as "probiotics." They are applied to prevent or to treat enteric disease in humans and animals (O'Sullivan et al., 1999).

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CHAPTER 1.2.6

Enterococcus

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Physiology and Genetics—General Introduction

Studies of enterococcal physiology have been conducted, for the most part, on members of two species, *Enterococcus faecalis* and *Enterococcus faecium*, with the exception of a few reports on *Enterococcus hirae*, which in many earlier publications was identified as *E. faecalis* or *E. faecium*. *Enterococcus faecalis*, recognized as a distinct species, is distinguishable from the *E. faecium* group, to which both *E. faecium* and *E. hirae* have been assigned on the basis of 16S rRNA sequences. Physiologically, *E. faecalis* can be distinguished from members of the *E. faecium* group in that the former can produce acid from glycerol, can ferment pyruvate, can utilize menaquinones as non-cytochrome electron carriers, and does not require exogenous folic acid for growth. *Enterococcus hirae* is physiologically distinguishable from *E. faecium* on the basis of the latter's ability to produce acid from L-arabinose (Devriese et al., 1993).

Most enterococcal genetic studies have involved strains of *E. faecalis*, a small number have involved *E. faecium*, and less than a handful have involved other enterococcal species, mostly antibiotic-resistant clinical isolates. Several plasmids of enterococcal origin have been studied in detail, including two types of conjugative plasmids: those that transfer via solid surface matings only and those that respond to *Enterococcus*-specific pheromones. Numerous nontransmissible plasmids, many of which are mobilizable by conjugative plasmids, also have been described, and some have been shown to be composites of two or more smaller plasmids. Several transposons, including Tn*916* (Franke and Clewell, 1981), the first conjugative transposable element to be described, have been identified in enterococcal isolates, and many have received considerable attention.

Until recently, the physiology and genetics of the enterococci have been totally separate fields. However, because of the recent completion of the nucleotide base sequences of the genomes of strains of two enterococcal species, *E. faecalis* (see The Institute for Genomic Research website) and *E. faecium* (see The Department of Energy Joint Genome Institute Web site), it should soon be possible to identify the genetic basis of all enterococcal physiological traits, including their mechanisms of regulation.

Physiology

General Physiological Traits

The enterococci, unlike the streptococci (once considered members of the same genus), are not confined to carbohydrates for their energy needs. In addition to 15 to greater than 30 different carbohydrates, depending on the species, a variety of other substrates may serve as energy sources, e.g., glycerol, lactic acid, malic acid, citric acid, α-keto acids, and the diamino acids (arginine and agmatine). As with the lactic acid bacteria in general, substrate-level phosphorylation is the primary mechanism of ATP generation. They lack most of the enzymes of the tricarboxylic acid (TCA) cycle, as well as typical electron transport chains. Some species, *E. faecalis* in particular, are not totally dependent on substrate-level phosphorylation for the production of energy, but can also produce a proton motive force via electron transport to drive oxidative phosphorylation.

The absence of catalase as well as a typical electron transport chain among the lactic acid bacteria is attributed to their inability to synthesize porphyrins. However, *E. faecalis* synthesizes a number of enzymes that function to rid the cell of toxic H_2O_2. Some strains of *E. faecalis* have been shown to develop catalase-like activities if grown aerobically in the presence of added hemin, and certain strains of *E. faecalis*, but not *E. faecium*, have also been reported to synthesize cytochromes if provided hemin. Despite the inability to synthesize cytochromes in the absence of added hemin, *E. faecalis* is capable of oxidative phosphorylation. Growth yields of *E.*

faecalis are much better when cultures are grown aerobically, rather than anaerobically. Molar ATP yields under aerobic conditions up to twice those obtained under anaerobic conditions (Whittenbury, 1978) have been reported.

The enterococci possess a variety of ATP-dependent, phosphoenolpyruvate (PEP)-dependent, symport and antiport systems for the transport of nutrients, various ions, and catabolic end products through the cellular membrane.

Carbohydrate Metabolism

All species of enterococci, with the exception of a few strains, produce acid from 15 carbohydrates or carbohydrate-containing compounds. These include *N*-acetyl glucosamine, amygdalin, arbutin, cellobiose, D-fructose, galactose, β-gentiobiose, glucose, lactose, maltose, D-mannose, methyl-β-D-glucopyranoside, ribose, salicin, and trehalose. One or more species have also been reported to produce acid from other sugars, or sugar alcohols, such as adonitol, L-arabinose, D-arabitol, L-arabitol, D-glycodextrin, dulcitol, gluconate, glycerol, inulin, 2-ketogluconate, D-lyxose, mannitol, melizitose, melibiose, α-methyl-D-glucoside, D-raffinose, sorbitol, L-sorbose, and xylitol (Devriese et al., 1993). Despite this large number of potential substrates, glucose and, to some extent, gluconate, mannitol and glycerol, have been the only substrates whose metabolism has received any significant attention.

Enterococcus faecalis can express the enzymes of all three of the major carbohydrate catabolic pathways, the Embden-Meyerhof-Parnas (EMP) or glycolytic pathway, the Entner-Doudoroff pathway, and the hexose monophosphate pathway (Sokatch and Gunsalus, 1957). The enterococci very likely ferment sugars primarily, if not exclusively, via the EMP pathway, and the major product of fermentation is lactate. When grown at the expense of glucose, *E. faecalis* also synthesizes at least the oxidative enzymes of the hexose monophosphate pathway, i.e., glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, and phosphoketolase. Whether transaldolase is synthesized under the same conditions is not known (Sokatch, 1960). On the basis of results of studies in which labeled glucose was used as the substrate, however, the primary product of glucose fermentation was shown to be lactic acid (Gibbs et al., 1955; Platt and Foster, 1957), suggesting that the EMP is utilized almost exclusively for the metabolism of this substrate. Although lactate is the primary product of glucose fermentation by this enterococcal species, fermentation balances consistently reveal the presence of acetate, formate and ethanol. Production of these three fermentation products increases, at the expense of lactate,

under more alkaline growth conditions (Wood, 1961).

The enzymes of the Entner-Doudoroff pathway are induced in the presence of gluconate, but are absent when *E. faecalis* cultures are propagated in the presence of glucose (Wittenberger et al., 1971). Lactic acid dehydrogenase of *E. faecalis* has an absolute requirement for fructose-1,6 *bis*-phosphate for activation, involving a conformational change in the enzyme (Wittenberger and Angelo, 1970). This glycolytic intermediate also regulates the activity of one of two phosphogluconate dehydrogenases synthesized by *E. faecalis*. An nicotinamide adenine dinucleotide phosphate (NADP)-dependent phosphogluconate dehydrogenase is inhibited by fructose-1,-6 *bis*-phosphate, but is unaffected by ATP, whereas a nicotinamide adenine dinucleotide (NAD)-dependent enzyme is insensitive to fructose-1,6-*bis*-phosphate, but is inhibited by ATP (Brown and Wittenberger, 1972).

Carbohydrate Transport— Phosphoenolpyruvate-dependent Phosphotransferase System

The transport of many carbohydrates metabolized by enterococci is mediated by the phosphoenolpyruvate-dependent phosphotransferase system (PTS). These include D-glucose, D-fructose, lactose, maltose, D-mannose, trehalose, sucrose, mannitol and *N*-acetylglucosamine (Huycke, 2002). Although gluconate is transported via a proton motive force (PMF)-driven proton symport mechanism in most Gram-positive bacterial species, this nutrient appears to enter the cell via the PTS in *E. faecalis* (Saier et al., 1993).

Enzyme I of the *E. faecalis* PTS, which is constitutively expressed, has a molecular weight of 70,000, with the native form existing as a dimer of two identical subunits. A single histidine residue is phosphorylated at the expense of PEP (Alpert et al., 1985). Phosphorylated Enzyme I transfers its phosphate to a constitutively expressed histidine-containing protein (HPr), the second phosphocarrier protein of the PTS. Also, HPr has been purified from *E. faecalis* (Deutscher et al., 1986b) and crystallized (Jia et al., 1993). The protein has a molecular weight of 9,438. The structure has been determined at a 1.6 Å resolution, and it has been shown that torsion-angle strain plays a direct role in the functioning of HPr. Like other HPr proteins of Gram-positive bacteria, the *E. faecalis* protein can be phosphorylated at a histidine residue (at position 15) at the expense of phosphorylated Enzyme I, or at serine-46 by an ATP-dependent HPr kinase, the latter abolishing phosphotransfer to sugar-specific Enzyme IIs (Reizer et al., 1989; Heng-

stenberg et al., 1993). The ATP-dependent HPr kinase gene has been cloned from *E. faecalis* (Kravanja et al., 1999) and expressed in *Escherichia coli*. The enzyme has a bifunctional activity, i.e., ATP-dependent HPr kinase activity, expressed at high levels of ATP, as well as a phosphatase activity, hydrolyzing serine 46-phosphorylated HPr (P-Ser-HPr) at low ATP levels. ATP-dependent phosphorylation of HPr by the HPr kinase/phosphatase enzyme is activated by, but does not require the presence of fructose-1,6 *bis*-phosphate. Among Gram-positive bacteria, P-Ser-HPr interacts with a catabolite control protein, CcpA, which in turn binds to operon regulating catabolite response element (CRE) sequences resulting in carbon catabolite repression (CRR). A *ccpA* homolog has been cloned from the chromosome of *E. faecalis* and sequenced (Leboeuf et al., 2000). In addition to being involved in the regulation of carbohydrate metabolism, the PTS is also a major player in the regulation of aerobic glycerol metabolism by the enterococci.

Mannitol is transported among both Gram-positive and Gram-negative bacteria via the PTS, and the mannitol EII/EIII permease components are the only enterococcal sugar-specific PTS components to have been studied in any detail. Generally among lactic acid bacteria, there are two inducible mannitol-specific PTS enzymes involved in the transport and phosphorylation of this carbon and energy source, a soluble Enzyme III^{mtl} (EIII^{mtl}) and a membrane-bound Enzyme II^{mtl} (EII^{mtl}). The latter actually is responsible for the transport across the cytoplasmic membrane and phosphorylation of the substrate. The next step in the utilization of mannitol is mannitol-1-phosphate dehydrogenase, which oxidizes the cytoplasmic mannitol phosphate to fructose-6-phosphate, which is further metabolized via the glycolytic pathway. The genes encoding EIII^{mtl} and mannitol-1-phosphate dehydrogenase have been cloned from *E. faecalis* (Fischer et al., 1991). The predicted amino acid sequence of EII-I^{mtl} was 45% similar to EIII^{mtl} of *Staphylococcus carnosus*, and 41% similar to the EIII^{mtl} domain of the *E. coli* EII^{mtl} enzyme. The predicted amino acid sequence of the *E. faecalis* mannitol-1-phosphate dehydrogenase, when compared to the same enzyme from *S. carnosus* and *E. coli*, was shown to share a high degree of similarity with the respective putative N-terminal NAD$^+$-binding domains. The mannitol-specific genes of *E. faecalis* appear to be organized in an operon in the order, *mtlA*, *orfX*, *mtlF*, *mtlD*, with *mtlF* and *mtlD*, encoding EIII^{mtl} and mannitol-1-phosphate dehydrogenase, respectively. The predicted protein of the *E. faecalis mtlA* gene shares some similarity to the C-terminal portion of the *E. coli* EII^{mtl}, but this similarity does not include the active center cysteine of the latter.

Pyruvate Metabolism

The final product of the oxidation of any carbohydrate via glycolysis is pyruvate, which in *E. faecalis* may be further metabolized via several enzymes or combinations of enzymes, dependent on environmental conditions. Under anaerobic conditions, the pyruvate is reduced primarily to lactate by lactic dehydrogenase (LDH). The LDH of *E. faecalis*, as well as *E. faecium*, is activated by fructose-1,6-*bis*-phosphate, owing to an alteration in the conformation of LDH, which results in a lowering of its apparent Km for both pyruvate and reduced nicotinamide adenine dinucleotide (NADH; Wittenberger and Angelo, 1970). Even under strictly fermentative conditions, although the enterococci are homofermentative, always some acetate, formate and ethanol are formed, the proportions of which increase with rising pH (Wood, 1961). Following a transition from anaerobic to aerobic conditions, the products of glucose metabolism change from predominantly lactate to acetate and CO_2 (Snoep et al., 1992b). The production of acetate may be the result of the activity of pyruvate formate lyase (PFL), pyruvate dehydrogenase (PDH), or possibly even a third alternative, an uncharacterized PDH-like activity (Yamazaki et al., 1976). The carboxyl of pyruvate may end up in CO_2 via PDH or in formic acid (HCOOH) via PFL. There is no CO_2 formed from HCOOH by enterococci because of the absence of formate-hydrogen lyase enzymes (Lindmark et al., 1969). The PFL of *E. faecalis* has been purified and shown to exchange formate with the carboxyl group of oxalacetate, α-ketoglutarate, and α-ketobutyrate, as well as with pyruvate (Lindmark et al., 1969). The enzyme is unstable in the presence of oxygen, and the only co-enzyme required for its activity is thiamine-pyrophosphate (thiamine-PP). Acetyl-CoA, formed via the activities of either PFL or a PDH, may be converted to acetate or ethanol, dependent on the oxidation reduction (O/R) ratios and energy needs of the cell, which in turn are controlled by environmental conditions and available nutrients and cofactors.

Pyruvate, in addition to being the product of glycolysis, or an intermediate in the metabolism of gluconate via the hexose monophosphate pathway, may also serve as an energy source for the growth of *E. faecalis*, but not *E. faecium* (Deibel and Niven, 1964c). Pyruvate fermentation may occur under both anaerobic and aerobic conditions, and when present as an energy source, lipoic acid is required for growth (Deibel, 1964a). Lipoic acid is an essential cofactor of activity of the PDH complex (Snoep et al., 1993). In addition to being required for growth at the expense of pyruvate as the sole energy source, lipoic acid added to a chemostat culture of *E.*

faecalis growing under limiting glucose + pyruvate concentrations results in a change in fermentation pattern that suggests activation of PDH (Snoep et al., 1993). Under glucose-limited conditions, a change from aerobic to anaerobic conditions results in a change in end products from acetate plus CO_2 to predominantly lactate. Addition of excess glucose to the culture also causes a switch to homolactic fermentation. However, addition of excess pyruvate to the limited glucose chemostat culture results in increases in both PDH and PFL activities (Snoep et al., 1992a). The PDH complex of anaerobically grown *E. faecalis* has been purified and characterized (Snoep et al., 1992b). It was found to resemble PDH complexes of other Gram-positive bacteria and eukaryotes, consisting of four polypeptide chains. Functions assigned to the chains include pyruvate dehydrogenase (E1α and E1β), dihydrolipoyl transacetylase (E2), and lipoamide dehydrogenase. Anaerobic growth of *E. faecalis* on pyruvate involves the activity of LDH, as well as PDH (Snoep et al., 1990). Under these conditions, the pyruvate is functioning as both an electron donor and an electron acceptor. The crystal structure of the 60-subunit dihydrolipoyl acyltransferase core of the *E. faecalis* PDH complex has been determined (Izard et al., 1999).

Alpha Keto Acid Metabolism

A lipoic acid-dependent α-keto acid dehydrogenase complex, separate from PDH, has been described in *E. faecalis*. This complex, designated "BKDH," was purified and shown to catalyze the oxidative decarboxylation of the branched-chain α-keto acids, α-ketoisocaproic acid (KIC), α-ketoisovaleric acid (KIV), and α-keto-β-methylvaleric acid (KMV) to the corresponding branched-chain acyl-CoAs (Rudiger et al., 1972). Subsequently, the *bkd* gene cluster, which encodes BKDH (*bkdDABC*), an acylphosphotransferase (*ptb*), and an acyl kinase (*buk*), was cloned from *E. faecalis* and characterized (Ward et al., 1999). The *bkdD*, *A*, *B* and *C* genes correspond to the E3, E1α, E1β, and E2 polypeptide chains, respectively, by analogy to the PDH complex. The acylphosphotransferase and acyl kinase convert the branched-chain acyl-CoAs to their corresponding free acids, isovalerate, isobutyrate, and methylbutyrate, with the generation of ATP via substrate level phosphorylation. Results of gene knockout studies showed that the *bkd* gene cluster is required for utilization of α-keto acids as energy sources. The *bkd* gene cluster is an operon controlled via a single promoter 5' of the *ptb* gene (Ward et al., 2000). Expression of the operon is inducible by KIC, KIV or KMV, with the latter being the strongest inducer. Expression is inhibited by the presence of carbohydrates metabolized via the EMP, such as glucose, lactose, or fructose, but not by gluconate, which is metabolized via the hexose monophosphate pathway (HMP). Such results suggest that repression of the *bkd* operon is due to phosphorylation of HPr at serine-47, which is dependent on fructose-1-6 *bis*-phosphate. An additional indication that the *bkd* operon is subject to catabolite repression via the PTS is the presence of a putative catabolite response element (CRE) in the promoter region (Ward et al., 2000). The α-ketoacid pathway is functional under both anaerobic and aerobic conditions, but under the former, a suitable electron acceptor, such as fumarate, must be present for the oxidation of NADH. In the absence of such an electron acceptor, the α-ketoacid itself may serve as a temporary electron acceptor, as evidenced by the presence of an extracellular reduced α-ketoacid intermediate during incubation under anaerobic conditions. Under aerobic conditions, oxygen serves as the electron acceptor.

Citrate Metabolism

Citrate utilization is initiated by a C_2-C_4 cleavage to acetate and oxaloacetate, catalyzed by citrate lyase. Acetyl-CoA is not produced in the C_2-C_4 cleavage. Pyruvate is produced by the decarboxylation of oxaloacetate. When citrate is the only source of energy, lipoate is required (Deibel and Niven, 1964c). The lipoate requirement has been interpreted as an indication that the energy-producing step involves the fermentation of pyruvate, possibly via PDH. *Enterococcus faecalis* strain FAIR-E 229, isolated from Cheddar cheese, metabolizes citrate in skim milk but does not utilize citrate if either glucose or lactose is also present in the growth medium (Sarantinopoulos et al., 2001). Thus, citrate is utilized when it is the sole source of energy, yielding acetate and formate as the primary end products, suggesting activity of PFL.

Malate Utilization

Under anaerobic conditions, *E. faecalis* grows slowly at the expense of malate as the sole source of energy (Deibel, 1964a). Growth may be enhanced by the addition of fumarate to an anaerobically growing culture, or by a switch to aerobic conditions, suggesting that either fumarate or oxygen can serve as an electron acceptor for the utilization of malate. A decarboxylating malate:NAD oxidoreductase (malic enzyme) has been purified from *E. faecalis* (London and Meyer, 1969a) and characterized (London and Meyer, 1969b). Synthesis of the enzyme is

unaffected by the presence of glucose; however, its activity is inhibited by fructose-1,6 *bis*-phosphate, 3-phosphoglycerate, and ATP. On the other hand, induction of the malate transport system (malate permease) has been shown to be subject to catabolite repression (London and Meyer, 1970). It has been speculated that malate carbon enters the EMP pathway at the level of pyruvate following decarboxylation (London and Meyer, 1970).

Glycerol Metabolism

Most species of enterococci, including *E. faecalis*, *E. faecium*, *E. casseliflavus* and *E. dispar*, are able to ferment glycerol under aerobic or microaerophilic conditions (Gunsalus and Sherman, 1943; Charrier et al., 1997; Huycke, 2002). Glycerol enters the cell via facilitated diffusion under all conditions, and lactate is the final product of its fermentation after entrance into the EMP pathway. However, the route to lactate is different in anaerobic versus aerobic or microaerophilic environments (Jacobs and VanDemark, 1960). Aerobically, glycerol is phosphorylated by glycerol kinase to α-glycerophosphate. Since the glycerol is phosphorylated immediately upon entrance into the cell, a concentration gradient is formed constantly, such that any glycerol in the external environment can continuously enter the cell. The α-glycerophosphate is oxidized to dihydroxyacetone-phosphate via L-α-glycerophosphate oxidase, a flavin adenine dinucleotide (FAD)-linked enzyme. The enzyme has been purified and characterized from a strain of *E. faecium* that produces it in high yield (Esders and Michrina, 1979). The enzyme exists as a dimer and contains FAD as a tightly bound cofactor, at two moles of FAD per mole of enzyme. Fructose-1-phosphate and fructose-6-phosphate, but not glucose-1-phosphate, glucose-6-phosphate, or fructose-1,-6 *bis*-phosphate, are inhibitory. Dihydroxyacetone-phosphate is further oxidized via the bottom half of the EMP pathway to pyruvate. Under aerobic conditions, lactate is the final end product accompanied by an accumulation of hydrogen peroxide.

Among the enterococci, only *E. faecalis* can utilize glycerol as a source of energy under anaerobic conditions (Gunsalus and Sherman, 1943). The first step in the anaerobic utilization of glycerol by *E. faecalis*, following facilitated diffusion into the cell, is oxidation to dihydroxyacetone, a reaction catalyzed by an NAD-linked glycerol dehydrogenase. Dihydroxyacetone kinase then catalyzes the phosphorylation of dihydroxyacetone to dihydroxyacetone-phosphate, which is further oxidized via the bottom half of the HMP pathway. It was noted early

(Gunsalus, 1947; Jacobs and VanDemark, 1960) that fumarate can accept electrons from the NADH formed during the anaerobic utilization of glycerol, with the production of succinate. Subsequently, this was shown to involve a simple electron transport chain comprised of glycerol dehydrogenase, a *b*-type cytochrome, and fumarate reductase. In this system, fumarate serves as the terminal electron acceptor, reduced to succinate by fumarate reductase (Gunsalus and Shuster, 1961). Recently, a strain of *E. faecalis*, RKY1, was described that could not ferment glycerol anaerobically unless fumarate was added to the growth medium (Ryu et al., 2001). This strain produced no detectable lactate from the fermentation of glycerol, instead producing succinate, acetate, CO_2 and formate. The strain also produced high amounts of succinate from the fermentation of glucose if grown in the presence of added fumarate.

Aerobic metabolism of glycerol by enterococci is highly regulated at the level of glycerol kinase, as is true for glycerol metabolism among Gram-positive and Gram-negative bacteria, in general. However, whereas allosteric inhibition of glycerol kinase by fructose-1,6 *bis*-phosphate is common among both Gram-positive and Gram-negative microorganisms (Deutscher and Sauerwald, 1986a; deBoer et al., 1986; Liu et al., 1994), as is regulation by the PTS, the actual components of the latter system involved in glycerol kinase regulation differ among the two groups of bacteria (deBoer et al., 1986; Deutscher and Sauerwald, 1986a; van der Vlag et al., 1994). Glycerol kinase is phosphorylated at the N-3 position of a histidyl residue by Enzyme I and HPr of the PTS at the expense of PEP, rendering it ten times more active than the unphosphorylated enzyme (Deutscher and Sauerwald, 1986a). The reaction is reversible in that phosphoglycerol kinase can transfer the phosphate group to HPr, which may occur if a PTS substrate, metabolized in preference to glycerol, is added to the medium, since the addition of such a substrate results in a predominance of the less active unphosphorylated glycerol kinase (Deutscher et al., 1993). The gene (*glpK*) encoding glycerol kinase has been cloned from *E. faecalis* and from *E. casseliflavus* (Charrier et al., 1997). Recombinant enzyme from both species, purified from *E. coli*, was phosphorylated in vitro at the expense of PEP in the presence of Enzyme I and HPr, resulting in a ninefold increase in activity. Both Enzyme I and HPr were required for phosphorylation. The site of phosphorylation on the glycerol kinase from *E. casseliflavus* was identified as His-232. Replacement of this residue by alanine, glutamate, or arginine eliminated the ability of the enzyme to be phosphorylated. The activity of the enzyme purified from

E. casseliflavus was inhibited by fructose-1,6 *bis*-phosphate.

Aerobic Lactate Utilization

A strain of *E. faecium*, with an ability to utilize lactate as a source of carbon and energy, has been described (London, 1968). Aerobic growth on lactate alone is concentration dependent, with a growth yield that is approximately 5% of that obtained with equivalent amounts of glucose. The presence of glucose and lactate in the growth medium results in enhanced growth yields relative to glucose alone. No lactate-specific growth factors could be identified. Lactate could not be utilized for growth under anaerobic conditions, even in the presence of added fumarate. A lactate-oxidizing enzyme, or enzyme system (lactate oxidase), is present in cultures grown aerobically on a number of hexoses, pentoses and trioses but appears to be repressed during growth in the presence of fructose. Lactate oxidase is also repressed during anaerobic growth. The enzyme(s) converts lactate to acetate and CO_2 with the consumption of oxygen. No hydrogen peroxide is produced in the reaction. *Enterococcus faecalis* oxidizes lactate 10–20 times faster in the presence of hematin than in its absence, and this increased rate of lactate oxidation is inhibited by the respiratory uncouplers carbonyl cyanide chlorophenylhydrazone (CCCP) and gramicidin D (Pritchard and Wimpenny, 1978). Such hematin-grown cells contain a membrane-bound cytochrome system (Ritchey and Seeley, 1976).

Amino Acid Catabolism

Only *E. faecalis* among the enterococci can utilize arginine, agmatine, and L-serine as sources of energy for growth (Deibel, 1964a). All enterococci can hydrolyze arginine, and approximately 50% of *E. faecium* strains can hydrolyze agmatine. Although not used as sources of energy, tyrosine and phenylalanine can be decarboxylated by the majority of enterococci.

The complete pathways for the utilization of arginine, the arginine deiminase (ADI) pathway, and the agmatine deiminase (AgDI) pathway, by *E. faecalis* have been elucidated (Simon and Stalon, 1982a; Simon et al., 1982b; Cunin et al., 1986). The pathways are similar, and each requires three enzymes. The first reaction is a deimination of the substrate to citrulline (in the ADI pathway) or carbamoylputrescine (in the AgDI pathway), by a substrate-specific deiminase, each yielding NH_3. The next step is phosphorolysis of citrulline by ornithine carbamoyltransferase, yielding carbamoylphosphate plus ornithine (in the ADI pathway), or of carbamoylputrescine by putrescine carbamoyl-

transferase, producing carbamoylphosphate plus putrescine (in the AgDI pathway). Finally, there is a carbamate kinase specific for each pathway that catalyzes the transfer of the carbamoylphosphate high-energy phosphate bond to ADP, yielding ATP, CO_2 and NH_3. The net energy yield of each pathway is 1 mole of ATP per mole of substrate. Induction of these enzymes is pathway specific, i.e., enzymes of the ADI pathway are induced only in the presence of arginine, and those of the AgDI pathway are induced only in the presence of agmatine. Whereas synthesis of the ADI pathway enzymes is repressed in the presence of glucose or fumarate, as well as during growth under anaerobic conditions (Simon et al., 1982b), synthesis of the AgDI pathway enzymes is repressed by either glucose or arginine (Simon and Stalon, 1982a).

Arginine enters the *E. faecalis* cell via an energy independent, arginine-inducible, arginine/ornithine antiporter (Poolman et al., 1987). Similarly, agmatine is transported into the cell by an agmatine-inducible agmatine/putrescine antiporter (Driessen et al., 1988). While the former system is specific for monovalent, positively charged amino acids, such as arginine, homoarginine and lysine, the latter appears to be specific for divalent positively charged diamines such as agmatine, and possibly also homoagmatine and cadaverine (Driessen et al., 1988).

Energy Metabolism

The enterococci derive most of their energy when grown anaerobically via substrate level phosphorylation. Sources of ATP are primarily carbohydrates, which are oxidized through the glycolytic pathway, and NAD is regenerated via the reduction of pyruvate to lactate. Growth conditions can alter the amounts of lactate produced, with concomitant changes in the amounts of acetate, ethanol and CO_2 made. Whereas at an acidic pH, the primary product of glycolysis is lactic acid, under alkaline conditions more ethanol, CO_2 and acetate are produced, with concomitantly greater yields of ATP from the production of acetate (Gunsalus and Niven, 1942; Graham and Lund, 1983). Unlike the streptococci, which rely exclusively on carbohydrate fermentation for their energy needs, the enterococci may generate ATP through the metabolism of a number of other substrates. The ATP produced by substrate-level phosphorylation is hydrolyzed by F_0F_1-ATPase to produce a proton potential for maintenance of cytoplasmic pH and for a variety of proton-coupled transport reactions. The maximum size of the proton potential that is generated is generally between -130 and -150 mV, while a potential of at least -200 mV is required for the reverse reaction, i.e., the synthesis of ATP by the F_0F_1-ATPase (Kakinuma, 1998). Thus,

among the enterococci, as is true of nonrespiratory bacteria, this ATPase does not appear to be a major contributor of cellular ATP.

The F_0F_1-ATPase of *E. hirae* was the first of the bacterial ATPases to be described (Abrams et al., 1960). The primary physiological role of this enzyme in enterococci appears to be the maintenance of cytoplasmic pH under acid environmental conditions. It also provides proton potential for proton-linked secondary transport systems. The operon encoding F_0F_1-ATPase activity has been cloned and sequenced (Shibata et al., 1992), and although the enzyme itself appears evolutionarily close to that of *Bacillus megaterium*, the order of genes appeared to be identical to that found in the corresponding operon of *E. coli* and nearly identical to that in *Streptococcus mutans*. The enzyme has an optimal activity at pH 6.0–6.5, has minimal activity at alkaline pHs, and unlike many other bacterial proton ATPases, is not inhibited by azide (Kakinuma, 1998). Not only is the activity of the enzyme optimized at acid pH, but also the amount of enzyme appears to increase. This was shown not to be due to increased transcription or to the synthesis of new enzyme subunits, but rather to an increase in the amounts of functional enzyme assembled on the cell membrane, suggesting that regulation is primarily posttranslational. *Enterococcus hirae* was shown to adapt to acid stress within a single generation in continuous culture, but de-adaptation occurred over several generations (Belli and Marquis, 1991).

Proton motive force can be generated in *E. faecalis* by excretion of lactic acid, the major end product of fermentation. Synthesis of ATP at the expense of this PMF was demonstrated with inside-out membrane vesicles. An influx of lactic acid into the inside-out vesicles resulted in the synthesis of ATP outside of the vesicles, which was inhibited by CCCP and by *N*-4, *N*-1-dicyclohexylcarbodiimide (DCCD; Simpson et al., 1983). However, more recent evidence indicates that in *E. faecalis* the concentration of free cytoplasmic lactic acid is balanced precisely with the proton electrochemical potential gradient over a wide range of pHs. At high external pH, there is a pool of tightly bound intracellular lactate. These data would suggest that transport of lactate to the outside of the cell is not an ATP-yielding event (Hockings and Rogers, 1997).

The first evidence for oxidative phosphorylation by enterococci was obtained when it was shown that the ATP could be synthesized via the oxidation of NADH by cell-free extracts of aerobically grown *E. faecalis* (Gallin and VanDemark, 1964). Subsequently, it was proposed that oxidative phosphorylation occurred in this species on the basis of molar growth yields (Smalley et al., 1968). From the demonstration

of flavins and naphthoquinones in this species (Baum and Dolin, 1963), it was postulated that oxidative phosphorylation would be at the level of NADH/flavin or via the oxidation of reduced naphthoquinone (Gallin and VanDemark, 1964). Earlier, the presence of an FAD-dependent NADH oxidase had been detected in extracts of aerobically grown *E. faecalis* that catalyzed the 4-electron reduction of oxygen to water (Hoskins et al., 1962). The NADH oxidase was purified (Schmidt et al., 1986; Ahmed and Claiborne, 1989), and it was shown that this enzyme was more closely related to flavoprotein NADH peroxidase of *E. faecalis* than to the flavoprotein monooxygenases, which also catalyze the four-electron reduction of oxygen (Ahmed and Claiborne, 1989).

Although the enterococci do not synthesize cytochromes because they cannot synthesize porphyrins, and were generally considered to be anaerobes, they do synthesize cytochromes if provided with hemin (Whittenbury, 1964). A naphthoquinone was isolated from a strain of *E. faecalis* and identified as 2-solanesyl-1,4-naphthoquinone, or demethylmenaquinone (Baum and Dolin, 1965). The naphthoquinone content of aerobically grown *E. faecalis* was approximately 1.6 times that of anaerobically grown cells. The presence of demethylmenaquinone in *E. faecalis* has also been demonstrated in *E. casseliflavus* and *E. galinarum*, but not in either *E. faecium* or *E. durans*. In addition to the demonstration that *E. faecalis* could make cytochromes in the presence of added hemin, it was also shown that oxygen could serve as an electron acceptor, allowing for higher growth yields and the synthesis of more ATP from glucose under aerobic conditions (Ritchey and Seeley, 1974). Subsequently, it also was shown that most strains of *E. faecalis* grown in the presence of hemin contained cytochrome-like respiratory systems, and it was proposed that they would have cytochrome-like NADH oxidase (Ritchey and Seeley, 1976). Strains of *E. faecium*, however, did not contain any cytochrome-like respiratory systems. The ability of *E. faecalis* to synthesize, in the presence of hematin, a functional cytochrome system in the cellular membrane was confirmed (Pritchard and Wimpenny, 1978). Membrane preparations from cells grown in the presence of hematin had higher levels of NADH oxidase activity, a *b*-type cytochrome, and two possible oxidase components (a cytochrome d_{630} and a CO-binding cytochrome). Evidence was also provided that the transport of electrons to oxygen by the hematin-dependent cytochrome system is coupled to proton translocation. A gene cluster has been identified in the genome sequence of *E. faecalis*, which shares similarity to the cytochrome *bd*-type respiratory oxidase operon, *cydABCD* of

Bacillus subtilis (Winstedt et al., 2000). Membranes from *E. faecalis* cells grown aerobically in the presence of hemin had absorption difference spectra characteristic of cytochrome *bd*. The *cydABCD* gene cluster of *E. faecalis* was cloned and expressed in *B. subtilis*, and the recombinant *B. subtilis* clone formed a spectroscopically detectable cytochrome *bd*. Furthermore, the cloned *cydABCD* gene cluster was shown to complement a cytochrome *bd*-deficient mutant of *B. subtilis*.

Fumarate appears to be the only electron acceptor available to enterococci for anaerobic respiration. The presence of fumarate reductase in *E. faecalis* has been reported (Deibel and Kvetkas, 1964b), which in addition to its constitutive expression by *E. faecalis* may be present in some strains of *E. faecium* (Aue and Deibel, 1967). Under anaerobic conditions, if fumarate reductase is present, greater amounts of acetate are produced, with concomitantly greater yields of ATP. Excess electrons, present because of the production of acetate rather than lactate, are transferred by NADH dehydrogenase to fumarate by fumarate reductase (Kakinuma, 1998).

Cation Transport

Cations are required for the activities of numerous enzymes, yet these ions are also toxic to cells if present at too high a concentration. Thus, all types of cells contain machinery necessary to regulate intracellular levels of cations, e.g., potassium and sodium, which are maintained at higher and lower levels, respectively, than are generally present in the environment. Many ion transport systems make use of the cell's proton potential for their activity. Since the enterococci lack respiratory chains, they are able to generate proton potential only via the hydrolysis of ATP, a reaction mediated by the proton-translocating enzyme, F_0F_1-ATPase. Consequently, for the enterococci, ATP plays a much more important role in the transport of ions through their membranes than is true of respiring bacterial genera. These microorganisms do synthesize, however, both proton- and ATP-dependent cation transport systems.

SODIUM TRANSPORT   Like the F_0F_1-ATPase of *E. hirae*, the first confirmed presence of a Na^+/H^+ antiporter was also provided through research on this microorganism (Kakinuma, 1987). The gene encoding this antiporter, *napA*, has been cloned (Waser et al., 1992), and its gene product, NapA, was shown to recognize Li^+, as well as Na^+, as a substrate (Strausak and Solioz, 1994). The presence of either cation in the environment will serve as a transcriptional inducer. A second mechanism of sodium extrusion involving a Na^+-ATPase has also been described in *E. hirae*

(Heefner and Harold, 1982). This sodium pump belongs to a subcategory (V-ATPases) of ion-translocating ATPases, which do not form enzyme-phosphate intermediates, as do the P-ATPases. This category encompasses the F-ATPases, i.e., F_0F_1-ATPases and the V-ATPases, a term derived from the proton pumps of acidic organelles, such as the vacuoles of fungi and plants, and the endosomes of animal cells. Unlike the F_0F_1-ATPase of *E. hirae*, the Na^+-ATPase exhibits maximal activity at pH 8.5–9.0, but exhibits no detectable activity at pH 6.0. It is stimulated by either Na^+ or Li^+, but catalytic activity absolutely requires Na^+ (Kakinuma, 1998). The V_0V_1-ATPase is encoded by the *ntpFIKECGABDHJ* operon (Murata et al., 1997), and all but *ntpH* and *ntpJ* are required for expression of the ATPase (Kakinuma et al., 1999b). The operon is induced by high intracellular concentrations of Na^+, by high pH, and when NapA is nonfunctional (Murata et al., 1996). A gene, *ntpR*, transcribed in the opposite orientation of the *ntp* operon, was originally thought to be involved in the regulation of that operon. However, interruption of *ntpR* had no effects on expression of the *ntp* operon. On the other hand, results from deletion studies indicated that an AT-rich track of DNA between -198 and -132 of the *ntp* operon is required for operon transcription. It has been speculated that this region is the binding site for a *trans*-activating protein involved in *ntp* transcription (Yasumura et al., 2002).

POTASSIUM TRANSPORT   Two systems involved in the uptake of potassium by *E. hirai*, KtrI and KtrII, as well as a potassium extrusion system, Kep, have been described. KtrI is the major K^+ uptake system under most conditions and requires both proton motive force and a high energy compound such as ATP. The system appears to be synthesized constitutively, and in addition to K^+, also recognizes Rb^+ (Kakinuma, 1998). First described in 1980 (Bakker and Harold, 1980), the KtrI system probably involves a K^+/H^+ symport, although this has yet to be demonstrated directly and is regulated by an ATP-dependent modification. It is active under neutral and acidic conditions, but inactive at alkaline pHs. The KtrII system requires neither ATP nor a membrane potential (Kobayashi, 1982), nor is it inducible by insufficient K^+ or repressed by excess K^+. It appears to be induced in the presence of excess intracellular Na^+ and requires the integral protein encoded by *ntpJ*, the last gene of the Na^+-ATPase-encoding *ntp* operon (Kakinuma et al., 1999b).

The Kep K^+/H^+ antiporter exports potassium ions against a concentration gradient in exchange for protons. The system is constitutively expressed, but functions only under

alkaline conditions (Kakinuma and Igarashi, 1999a).

More recently, an energy-dependent potassium uptake system with a low affinity for potassium was described that functions at pH 10.0 (Kawano et al., 2001). This system was discovered in a mutant of *E. hirae*, defective in both KtrI and KtrII, that did not grow at pH 10 in the presence of low concentrations of potassium (<1 mM) but grew well in the presence of 10 mM KCl.

CALCIUM AND IRON TRANSPORT Calcium is extruded from *E. hirae* by a primary active transport mechanism that can establish a 30:1 out/in gradient (Kobayashi et al., 1978). The system requires ATP, but apparently functions in the absence of proton potential. To date, no calcium-specific uptake system has been described for the enterococci.

Virtually no work has been done on mechanisms of iron transport among the enterococci, although siderophores have been detected in members of this genus. In one study (Lisiecki et al., 2000), 70 strains representing 16 enterococcal species were shown to have linear trihydroxamate or citrate hydroxamate siderophores. In another study, 6 of 6 *E. faecium* and 2 of 6 *E. faecalis* strains were able to utilize siderophores provided by Gram-negative bacterial species, and to some extent, siderophores from other Gram-positive bacterial species (Szarapinska-Kwaszewska and Mikucki, 2001). Enterococci clearly can utilize iron from numerous environmental sources, since strains of several species were shown to grow under conditions of iron depletion in vitro if provided with such sources of iron as bovine hemoglobin, hemin, lactoferrin, transferrin, ovotransferrin, horse myoglobin, ferritin, or cytochrome *c* (Sobis-Glinkowska et al., 2001).

COPPER TRANSPORT *Enterococcus hirae* (ATCC9790) encodes an operon, *cop*, the products of which maintain copper homeostasis. The operon consists of four genes. The *copA* and *copB* genes encode P-type ATPases, CopA and CopB, whereas *copY* and *copZ* encode a copper-responsive repressor, CopY, and a copper chaperone, CopZ (Wunderli-Ye and Solioz, 1999). Cop A is responsible for copper uptake under conditions of copper limitation. CopB is an extrusion ATPase that exports excess copper from the cytoplasm, rendering the cell copper-resistant. Regulation of the *cop* operon is biphasic, with low-level induction expressed at copper concentrations of 10 μ M and maximum induction at concentrations of 2 mM. Maximum induction is also seen in the presence of μ M concentrations of the fortuitous inducers, Ag^+ and Cd^+. Mutations in *copY* result in constitutive expression of the *cop* operon, suggesting that CopY is a repressor. CopZ is a small (69-aa [amino acid]) protein and a member of a family of copper chaperones that includes MerP (a mercury binding protein), ATX1 (a yeast copper chaperone), and HAH1 (a human copper chaperone).

Oxygen Metabolism by Enterococci

The enterococci produce toxic H_2O_2 and/or superoxide, O_2^- under a variety of environmental and nutritional conditions (Falcioni et al., 1981; Pugh and Knowles, 1983; Huycke et al., 1996; Winters et al., 1998). These bacteria can synthesize several enzymes that remove such toxic metabolic biproducts. *Enterococcus faecalis* may synthesize a flavoprotein NADH peroxidase, as well as a heme-containing catalase, when grown aerobically. The latter also requires the addition of hematin to the growth medium (Pugh and Knowles, 1983). More recently, a chromosomal gene, *katA*, was identified in the sequenced chromosome of *E. faecalis* strain V583, and the purified enzyme synthesized by this strain was shown to belong to a family of monofunctional catalases (Frankenberg et al., 2002). *Enterococcus faecalis* produces an NADH oxidase that catalyzes a four-electron reduction of oxygen to water without formation of any O_2^- or H_2O_2 (Schmidt et al., 1986), as well as an oxygen-inducible superoxide dismutase (Britton et al., 1978).

Genetics

Introduction

The beginnings of enterococcal genetics can be traced to the first report of an association of antibiotic resistance with plasmid DNA in an isolate of *E. faecalis* (Courvalin et al., 1972). This was followed by a description of high frequency conjugative transfer of a hemolysin/bacteriocin trait from one strain of *E. faecalis* to another (Tomura et al., 1973), although no evidence for the involvement of plasmid DNA or any type of transmissible genetic element was presented. By the following year, resistance of a strain of *E. faecalis* to erythromycin and tetracycline was correlated with the presence of two specific plasmids (Courvalin et al., 1974), and the first definitive description of plasmid-mediated antibiotic resistance transfer between strains of *E. faecalis* by a mechanism that resembled conjugation appeared (Jacob and Hobbs, 1974). Prior to these studies, there had been only one unconfirmed report of gene transfer in this genus (Raycroft and Zimmerman, 1964). From the mid-1970s onward, interest in the genetics of the enterococci has continued to intensify, to the extent

that there have been six American Society for Microbiology (ASM)-sponsored international conferences on streptococcal genetics, major sessions of which have been devoted to the enterococci. Papers describing many of the presentations from five of the six conferences have been published (Schlessinger, 1982; Ferretti and Curtiss, 1987; Dunny et al., 1991b; Ferretti et al., 1995; Yother et al., 2002). More recently, four chapters in a book on the genus, *Enterococcus* (Gilmore et al., 2002), were devoted to different aspects of enterococcal genetics.

To date, there still are no reports of transduction in this genus, and natural competence for transformation has never been observed among enterococci. However, numerous plasmids have been shown to occur naturally in members of this genus, and two types of plasmid-mediated conjugation have been described, one of which is unique and apparently specific to enterococci. Plasmid replication and maintenance functions have been examined in some of these plasmids, as have the antibiotic resistance and virulence traits they encode. A number of transposable elements, including conjugative transposons, have been described. Finally, the sequencing of the genomes of two enterococcal species, *E. faecalis* (see The Institute for Genomic Research Web site) and *E. faecium* (see The Department of Energy Joint Genome Institute Web site), make possible the association of individual genes with specific functions, as well as the discovery of new genes whose functions remain to be determined.

Plasmids

A large number of plasmids, ranging in size from <5 to >100 kb, have been detected in and/or isolated from strains of *Enterococcus* spp., mostly *E. faecalis* (Clewell, 1981; Showsh et al., 2001). Enterococcal isolates may harbor as many as five or more distinct plasmid species (Clewell, 1981; Atkinson et al., 1997). Plasmids replicate via two basic mechanisms, rolling circle replication (RCR) and θ-replication (Janniere et al., 1993). On the basis of the ubiquity of RCR plasmids among Gram-positive bacterial species in general (Gruss and Ehrlich, 1989; Khan, 1997), one might expect that many of the smaller plasmids present in members of the genus *Enterococcus* would replicate via a rolling circle mechanism. As yet, no naturally occurring RCR plasmid has been described in enterococci. However, the tetracycline resistance plasmid, pAMα 1, originally isolated from *E. faecalis* strain DS5 (Clewell et al., 1974), was shown to be a composite of two replicons, one of which, pAMα 1Δ 1, is identical to the 4.6-kb RCR plasmid, pBC16, from *Bacillus cereus* (Gruss and Erlich, 1989). The conjuga-

tive plasmid, pJH1 from *E. faecalis* strain JH1 (Jacob and Hobbs, 1974), was subsequently shown to be a composite plasmid consisting of at least two replicons, one of which was highly related to pAMα 1Δ 1 (Banai et al., 1985; Perkins and Youngman, 1983). Thus, although RCR plasmids have not been shown to exist as independent replicons in enterococci, they can in fact replicate quite stably in members of this genus, as evidenced by the number of RCR-based vector molecules that have been constructed and used for recombinant DNA studies in enterococci (Weaver et al., 2002). Two types of -replicating plasmids are common among the enterococci, pAMβ 1-like molecules, the so-called "Inc18 plasmids" (Janniere et al., 1993), and the pheromone-responsive plasmids (Clewell, 1999).

INC18 PLASMID REPLICATION pAMβ 1 was first isolated from *E. faecalis* strain DS5, which was also the source of two other well-studied enterococcal plasmids, pAMα 1 and pAD1 (Clewell et al., 1974). pAMβ 1 is closely related to several plasmids originally isolated from various species of *Streptococcus* and a number of plasmids from enterococcal isolates (Janniere et al., 1993). These plasmids range in size from about 25 to 30 kb, with copy numbers that range from 10 to 15 per chromosome equivalent. pAMβ 1 mediates erythromycin resistance (Clewell et al., 1974) due to the presence of an *ermB* determinant (Roberts et al., 1999) encoding resistance to the MLS group of antibiotics (macrolides, lincosamides, and streptogramin B). It was used to demonstrate the transformability by plasmid DNA of naturally competent strains of streptococci (LeBlanc and Hassell, 1976; LeBlanc et al., 1978a), and it was also shown to transfer to, and replicate in, several species of streptococci, if donor and recipient cells were forced together on a solid surface (LeBlanc et al., 1978b). Subsequently, pAMβ 1 and related plasmids have been shown to possess a very broad host range, i.e., the ability to transfer and replicate in a large number of Gram-positive bacterial species (Clewell, 1981; Horaud et al., 1985).

The replication of the Inc18 plasmids has received considerable attention (Bruand et al., 1993; Bruand and Ehrlich, 1998). Early studies narrowed the region of the 26.5-kb plasmid, pAMβ 1, required for replication to less than 3 kb (LeBlanc and Lee, 1984). It has since been shown to replicate via a unique type of mechanism (Bruand et al., 1993; Janniere et al., 1993). A transcript is synthesized that begins at the promoter of the initiation gene, *repE*, and proceeds through the replication origin. The transcript is processed, possibly by an RNase function of the RepE protein, creating a 10-nucleotide primer for the host DNA Pol I at the 3′ end of the

original transcript, and the 5′ end is released. Replication by Pol I continues for approximately 150 bp where an *ssiA* site is exposed for priming of the lagging strand via the product of *priA*. At this point, Pol I is replaced by DNA Pol III for continued synthesis of both the lagging and leading strands in the direction away from *repE*. RepE functions as the replication initiator by binding to double-stranded pAMβ 1 at the origin of replication, which causes bending and melting, resulting in a single-strand bubble at the origin. RepE also possesses strong, nonspecific single-strand binding activity that leads to the binding of additional RepE molecules at the bubble, which in turn results in the extension and stabilization of the single-strand region at the origin (LeChatelier et al., 2001). Also, RepE is both essential and rate-limiting for replication of pAMβ 1. Its synthesis is very tightly regulated by two mechanisms, negatively by a repressor protein, CopF, encoded 5′ of *repE*, and by counter-transcript-driven transcriptional attenuation mediated by an antisense RNA molecule transcribed from the opposite strand at the 5' end of *rep*, which causes termination of *rep* transcription (LeChatelier et al., 1996). Inc18 plasmids encode resolvase/invertase proteins (RIs), which appear to play a role in plasmid replication, partition or both. Such a protein, designated "Resβ," is encoded by pAMβ 1, and is involved in plasmid partition through its ability to convert dimers or higher oligomeric forms to monomers. Resβ binds to a target on the plasmid that is located approximately 200 bp 3′ of the origin of replication. When bound to the target, it functions as a roadblock preventing continued elongation by Pol I. Consequently, a replication intermediate is formed consisting of a 200-bp D-loop which, as a single-stranded form, contains a primosome assembly site, *ssiA*, at which the DNA primase, DNA helicase, and Pol III are loaded for continued replication (Alonso et al., 1996).

PHEROMONE-RESPONSIVE PLASMID REPLICATION
The pheromone-responsive plasmids are a group of enterococcal replicons that are self-transmissible at high frequency in broth only to other enterococci (Wirth, 1994; Dunny and Leonard, 1997; Clewell, 1999). The pheromone-responsive plasmids described to date range in size from 37 to 91 kb, are maintained at very low copy numbers (2–4 per chromosome equivalent), and with one exception, pHKK100 from *E. faecium* (Handwerger et al., 1990), have all been detected in isolates of *E. faecalis*. The first of these plasmids to be described was pAD1 (Dunny et al., 1978), and it, together with pCF10 (Dunny et al., 1981), has been studied in the greatest detail. The basic replicons of pAD1 (Weaver et al., 1993), pCF10 (Hedberg et al., 1996), pPD1 (Fujimoto

et al., 1995), and pAM373 (De Boever et al., 2000) have been identified. Equivalent genes have been identified in the basic replicons of all four plasmids with the designations for pAD1 being *repA*, *repB*, *repC* and *par* (different designations have been assigned to the corresponding homologs of pCF10; Hedberg et al., 1996), with iterons located on either side of the *repBC* operon. The *repA* gene encodes a replication initiator protein that falls within a family of Gram-positive bacterial plasmid initiator proteins (Hirt et al., 1996; Berg et al., 1998; Kearney et al., 2000). Within the central region of *repA* is a repeat structure that serves as the replication origin of pAD1 (Weaver et al., 2002), as well as other plasmids of Gram-positive bacteria (Gering et al., 1996; Tanaka and Ogura, 1998). This same region of pAD1 also functions as the origin of pheromone-mediated transfer of pAD1 (An and Clewell, 1997).

Transposon insertions in *repB* resulted in elevated copy numbers of pAD1 (Ike and Clewell, 1984), suggesting that this gene may be involved in maintenance of plasmid copy numbers. However, the likelihood that *repBC* is an operon would suggest that this result could also be due to a polar effect on *repC*. The general structure of *repBC* and its location between repeat sequences would suggest a relationship to plasmid *par* loci associated with plasmid partitioning (Gerdes et al., 2000).

The function of *par* in pAD1, and to a lesser extent pCF110, has been studied in some detail (Weaver et al., 1994; Weaver et al., 1996; Weaver et al., 1998). The *par* locus is not required for plasmid replication, but functions to insure stable inheritance of the plasmid via a post-segregational killing (PSK) mechanism. The system consists of two RNA transcripts that are synthesized convergently from opposite ends of the *par* locus and terminate at the same bidirectional transcriptional terminator, so that they have at their respective 3′ ends, complementary stem-loops. Each transcript also has the same repeat sequence at its 5′ end, such that the shorter transcript, RNA II (~145 nucleotides), is totally complementary to the longer transcript, RNA I (~250 nucleotides), but only at the 5′ and 3′ ends of the latter. Within RNA I is a gene, *fst*, that encodes a 33-aa peptide, Fst. Translation of *fst* from the RNA I transcript is normally inhibited in a pAD1-containing cell owing to the binding of the antisense molecule, RNA II, to RNA I at its 5′ end, which results in an inhibition of ribosome binding. Both RNA I and RNA II are transcribed by pAD1-containing cells of *E. faecalis*, such that Fst is never present. However, if cells in a population lose the plasmid, *fst* can be translated in plasmid-free cells because RNA II has a much shorter half-life than RNA I. Synthesis of Fst, which is an enterococcal toxin,

results in cell killing. Normally, in plasmid-containing cells, RNA II functions as an antitoxin by preventing the synthesis of the toxin. Recent data suggest that Fst compromises the integrity of the *E. faecalis* cell membrane, which is accompanied by cessation of all macromolecular synthesis and cell growth (Weaver et al., 2003).

Transposons

Tn3 Family of Transposons The first transposon identified in any enterococcal isolate was Tn*917*, a 5.4-kb erythromycin resistance element present on pAD2, one of two plasmids harbored by *E. faecalis* DS16 (Tomich et al., 1980). Both transposition and resistance were shown to be inducible by erythromycin, with relocation to pAD1 being increased by an order of magnitude by the presence of the antibiotic. A second transposon, Tn*3871*, very similar to Tn*917*, was identified on pJH1 from *E. faecalis* strain JH1 (Banai and LeBlanc, 1984), and Tn*917*-like elements were subsequently shown to have been disseminated among enterococcal isolates of both human and animal origin (Rollins et al., 1985). Results of studies of Tn*917* in *Bacillus subtilis* (Perkins and Youngman, 1984), as well as analyses of its nucleotide base sequence, and the predicted amino acid sequences of six identified open reading frames (ORFs; Shaw and Clewell, 1985), placed this enterococcal transposon in the Tn*3* family of transposons (Lett, 1988). Another enterococcal transposon belonging to the Tn*3* family is Tn*1546*, a 10.9-kb element encoding resistance to vancomycin, isolated from a strain of *E. faecium* (Arthur et al., 1993). Results from a recent study (Willems et al., 1999) have shown that Tn*1546* has undergone a large number of changes in vancomycin resistant enterococci (VRE). In this study, the *vanA*-encoding transposons present in 97 enterococcal isolates of human and animal origin were examined. A total of 22 different Tn*1546*-like elements were identified containing point mutations, insertions of IS elements, and deletions. Most of the transposons (76%) contained 1–3 copies of IS*1216V*. Insertions of IS*1251*, and of an IS*1216V*-IS*3*-like element were also detected.

Composite Transposons A type of transposon commonly found in enterococci is the composite transposon (Snyder and Champness, 1997). These transposons are characterized by the bracketing of genes on the host chromosome, a plasmid, or a pre-existing transposon, by two IS elements of the same type, thereby forming a larger transposon. The first composite transposon to be described in an enterococcal isolate was Tn*5281*, present on a conjugative plasmid, pBEM10, from a gentamicin-resistant strain of

E. faecalis (Hodel-Christian and Murray, 1991). Tn*5281* is very similar, if not identical, to the 4.7-kb staphylococcal gentamicin-resistance transposons, Tn*4001* and Tn*4031*, and contains at its ends the IS element, IS*256*. All of these transposons carry the bifunctional *aacA-aphD* gene (Ferretti et al., 1986) that mediates resistance to gentamicin, as well as other aminoglycoside antibiotics. Not all gentamicin resistant enterococci that encode *aacA-aphD* carry this gene on Tn*5281* (Hodel-Christian and Murray, 1992). Although three isolates from two different countries were shown to contain Tn*5281*-like transposons, two other isolates were shown to contain a transposon more like a Tn*4001* hybrid transposon with three copies of IS*257*, and which is no longer mobile. The *aacA-aphD* determinant of yet a fifth strain could not be associated with either type of transposon. A much larger gentamicin-resistance composite transposon (27 kb) designated "Tn*924*," was detected in an *E. faecalis* clinical isolate from Canada (Thal et al., 1994). This transposon was flanked by IS*257*-like elements.

IS*256* appears to be common among enterococci, even strains that are susceptible to gentamicin. In a study of more than 100 clinical enterococcal isolates (85 *E. faecium* and 15 *E. faecalis*), 85% were shown to contain copies of IS*256* (Rice and Thorisdottir, 1994). Nearly half of the isolates examined were susceptible to gentamicin, and these strains contained between one and six copies of Tn*256*. All but two of the Tn*256*-containing isolates were resistant to one or more of the antibiotics tested, which included, in addition to gentamicin, ampicillin, streptomycin and vancomycin. The presence of multiple copies of an IS element in enterococci has been demonstrated, and this situation could result in the formation of composite transposons encoding multiple antibiotic resistance (Rice et al., 1995). A strain of *E. faecalis* was shown to carry a 26-kb composite transposon comprised of a Tn*4001*-like element flanked by IS*256*, as well as an erythromycin-resistance determinant flanked by one of the former IS*256* sequences, plus a third copy of this IS element. Even larger multiple antibiotic-resistance composite transposons have been described. For instance, Tn*5385* is a 65-kb genetic element detected in the chromosome of an *E. faecalis* clinical isolate (Rice and Carias, 1998a). It contains genes encoding resistance to gentamicin, erythromycin, streptomycin, tetracycline/minocycline, penicillin (due to β-lactamase activity), and resistance to mercury, and is comprised of at least four previously described transposons, two of enterococcal origin (Tn*5381* and Tn*5385*) and two of staphylococcal origin (Tn*4001* and Tn*552*). These transposons are linked by copies of IS*256*, 20019 IS*257* and

IS*1216*; copies of the latter have been shown to be present at the ends of Tn*5385*.

Tn1*547* is a somewhat unique 64-kb element identified in a strain of *E. faecalis*, which encodes the entire *vanB* gene cluster, and has two different though distantly related IS elements at its ends (IS*256* and IS*16*; Quintiliani and Courvalin, 1996). Despite the presence of different IS elements, Tn*1547* was mobile.

CONJUGATIVE TRANSPOSONS The transfer of tetracycline resistance from one strain of *E. faecalis* to another via a mechanism that required cell-to-cell contact and could occur in the absence of plasmid DNA was described for the first time in 1981. Transfer of the tetracycline-resistance element, as well as its integration into chromosomal or plasmid DNA was *recA*-independent. The conjugative transposon responsible for both transfer and transposition was designated "Tn*916*" (Franke and Clewell, 1981). Subsequently, conjugative transposons have been discovered in both Gram-positive and Gram-negative bacterial genera, and numerous reviews have appeared in the literature. A partial list is included here (Clewell and Flannagan, 1993c; Scott, 1993; Salyers et al., 1995; Scott and Churchward, 1995; Lyras and Rood, 1997; Smith et al., 1998; Churchward, 2002). Transfer-specific aspects of Tn*916* will be covered elsewhere in this chapter, and only those functions associated with transposition will be discussed here. Tn*5* insertional mutagenesis was used to decipher the genetic organization of the transposon, permitted a separation of conjugative transposition from intracellular transposition, and identified regions of the element associated with excision, which is required for both conjugation and transposition (Senghas et al., 1988). It was discovered that Tn*916* forms a covalently closed intermediate structure upon excision from a host genome or plasmid (Scott et al., 1988). Excision and covalently closed circular intermediate formation occurred in *E. coli* following its cloning on an *E. coli* plasmid vector. Intermediate structures purified from *E. coli* could be used to transform *Bacillus subtilis*, and these transformants were able to donate Tn*916* to a strain of *Streptococcus pyogenes*. Excision of Tn*916* includes noncomplementary base pairs at each end of the transposon that form a heteroduplex at the joint of the circular intermediate. When the transposon inserts into a new target, those base pairs are inserted into the target DNA by a reversal of the excision process (Caparon and Scott, 1989). The complete sequence of Tn*916* was published in 1994, which confirmed a size of just over 18 kb and permitted the identification of 24 ORFs (Flannagan et al., 1994). Previously identified excision, *xis*, and integration, *int*, loci were assigned to specific ORFs, and one ORF was shown to be required for conjugation.

The Int protein of Tn*916* contains two DNA binding domains, one at its C-terminus, which binds to the ends of the transposon, as well as to the target DNA, and one at its N-terminus, which binds to sequences internal to the transposon, but close to each end (Lu and Churchward, 1994). It was postulated that the existence of the two DNA binding sites on Int provided a mechanism for insuring that only correctly aligned transposons and target molecules would undergo recombination. Results of additional studies showed that the C-terminal DNA binding domain of Int exhibited different binding affinities for different target sites, and that these affinities correlated with observed frequencies of transposon insertion at those sites (Lu and Churchward, 1995). Xis protein also binds near the ends of Tn*916*, close to direct repeat sequences associated with Int binding (Rudy et al., 1997). Both Int and Xis are required for Tn*916* excision (Marra and Scott, 1999). The Int protein is involved in both strand cleavage and joining reactions and is also essential for transposon integration (Jaworski et al., 1996).

Conjugation

The transfer of chloramphenicol resistance between strains of *E. faecalis*, by a mechanism that required cell-to-cell contact, was first reported in 1964 (Raycroft and Zimmerman, 1964). However, the donor strain was a chloramphenicol-resistant mutant of *E. faecalis* and was thought to transfer this mutant phenotype to recipient cells. No conjugative genetic element was ever identified, and the results were never confirmed in the literature. Approximately nine years later, a paper was published that reported the high frequency conjugative transfer between enterococci and the ability to produce bacteriocin (Tomura et al., 1973). Although the genetic element responsible for transfer was not identified, the transfer of this type of lytic activity from strains of *E. faecalis* has been confirmed numerous times since. The following year, plasmid-mediated, multiple antibiotic-resistance transfer between strains of *E. faecalis* at high frequency in broth culture was clearly documented (Jacob and Hobbs, 1974), as was the high frequency transfer of plasmid-mediated bacteriocin and hemolysin production as well as bacteriocin resistance, a year later (Jacob et al., 1975). Jacob and coworkers clearly demonstrated that two different large plasmids from the same strain of *E. faecalis* strain JH1 were transmissible: pJH1 encoded resistance to four different antibiotics, and pJH2 encoded hemolysin production and bacteriocin resistance. They also identified

another transmissible hemolysin/bacteriocin plasmid from a second donor strain.

PHEROMONE-RESPONSIVE BROTH MATING It was shown within three years that the conjugative transfer described by Jacob and colleagues in 1975 was mediated by plasmids that respond to a substance, termed "sex pheromone," which was produced by recipient strains that stimulated the formation of mating cell aggregates (Dunny et al., 1978). To date, at least 20 different pheromone responsive plasmids from 14 different enterococcal isolates have been isolated and characterized to some extent (Clewell and Dunny, 2002). All but one of these plasmids, pHKK100, from a strain of *E. faecium* (Handwerger et al., 1990) has been from *E. faecalis*. The pheromone-response mechanism of plasmid transfer has been studied in considerable detail, and several reviews describing it have been published (Clewell and Weaver, 1989; Dunny, 1990; Dunny, 1991a; Clewell, 1993a; Clewell, 1993b; Clewell, 1999; Wirth, 1994; Dunny et al., 1995; Wirth et al., 1996; Dunny and Leonard, 1997; Maqueda et al., 1997). This mechanism will be described briefly below.

Plasmid-free strains of *E. faecalis* excrete several chromosome-encoded small peptides (pheromones) that induce a clumping response by strains harboring one or more pheromone-responding plasmids. A potential donor cell responds to a specific pheromone by synthesizing plasmid-encoded aggregation substance (AS), which in turn binds to enterococcal binding substance (EBS) on the surfaces of both donor and recipient cells. Binding substance is at least in part composed of lipoteichoic acid. It has been suggested (Dunny et al., 1995) that the donor-recipient cell contacts formed as a result of AS-EBS binding in some manner allows for the formation of a channel through which plasmid DNA can transfer from the donor cell to the recipient cell. Nearly all pheromone-responsive plasmids encode surface exclusion determinants that prevent, or at least drastically lower the frequency of donor-to-donor plasmid transfer. Pheromone-responsive plasmids also encode inhibitors of the pheromones that induce their response. Thus, once a recipient cell receives a specific pheromone-responsive plasmid, it no longer excretes that pheromone, although it will continue to synthesize and excrete pheromones specific for other pheromone-responsive plasmids. Pheromones and pheromone inhibitors are small peptides composed of 7–8 hydrophobic amino acids. Both are synthesized as precursor molecules requiring processing.

Four pheromone-responsive plasmids, pAD1, pCF10, pAM373 and pPD1, have received considerable attention. The entire nucleotide base sequences of pAD1 (Francia et al., 2001) and pAM373 (De Boever et al., 2000) have been published, and the sequences of large segments of pCF10 have also been published (Kao et al., 1991; Hedberg et al., 1996), with the remainder soon to follow (Clewell and Dunny, 2002). Determinants that share the same or similar functions also share a good deal of sequence homology, and the arrangement on each of the four plasmids of groups of homologous functions, such as replication, pheromone response, transfer, etc., is also similar. Regulation of the pheromone response of plasmids pAD1 (Clewell, 1999) and pCF10 (Dunny and Leonard, 1997) has been examined in considerable detail. Basically, the synthesis of transfer-specific genes by each plasmid is under the regulation of two promoters. One, P_0, regulates the synthesis of an inhibitor of plasmid-specific pheromone and is negatively regulated by the product of a consititutively synthesized protein, TraA of pAD1 and PrgX of pCF10. A second promoter, P_a, from which antisense transcripts are synthesized, further insure that expression from P_0 does not proceed through two transcriptional terminators just 5' of two ORFs that encode two positive regulators of transcription and/or translation of conjugation-specific functions. Basically, pAD1-specific or pCF10-specific pheromone produced by a potential recipient interacts with TraA or PrgX, respectively, inactivating negative regulator. The resulting release in the negative control of P_0 allows read-through past both transcriptional terminators into the genes that encode positive regulation of transfer-specific genes.

PLASMID-MEDIATED SOLID-SURFACE MATING The erythromycin resistance plasmid, pAMβ 1, from *E. faecalis* strain DS5 (Clewell et al., 1974) was used to demonstrate the transformability of competent strains of streptococci by plasmid DNA (LeBlanc and Hassell, 1976; LeBlanc et al., 1978a). Subsequently, the ability of streptococcal transformants containing pAMβ 1 to transfer the plasmid to other species of *Streptococcus* by a mechanism that required cell-to-cell contact was demonstrated (LeBlanc et al., 1978b). Unlike the pheromone-responsive plasmids, pAMβ 1 is transmissible only if donor and recipient cells are forced together on a solid surface, i.e., either directly on an agar-based medium, or by collection of donor and recipient cells on a filter membrane followed by incubation of the filter on the surface of such media. The transmissibility of pAMβ 1 from its strain of origin, *E. faecalis* strain DS5, could not be demonstrated, likely because of the presence of pheromone-responsive plasmids in this strain and because another such plasmid, pAD1, was shown to inhibit its transfer (Clewell et al., 1982). Numerous plasmids from enterococci and various streptococcal species, many exhibiting considerable

homology to pAMβ 1, have been shown to mediate their own transfer by solid surface mating to a broad spectrum of Gram-positive bacteria, including most species of streptococci (Clewell, 1981; Horaud et al., 1985). However, although a region of pAMβ 1 (LeBlanc and Lee, 1984) and several regions of the closely related plasmid, pIP501 (Evans et al., 1985; Krah and Macrina, 1989), required for conjugation have been identified, the actual mechanism of transfer has never been elucidated.

Transposon-mediated Solid-surface Mating Conjugative transfer in the absence of any plasmid DNA was first demonstrated in enterococci (Franke and Clewell, 1981) and chromosome-borne transposon, Tn*916*, was shown to mediate this transfer. As in the transfer mediated by pAMβ 1-like plasmids, transposon-mediated conjugation also occurs at detectable frequencies only on solid surfaces. Although a large number of conjugative transposons have now been described in both Gram-positive (Clewell and Flannagan, 1993c; Lyras and Rood, 1997) and Gram-negative (Salyers et al., 1995; Smith et al., 1998) bacteria, the most thoroughly studied have been Tn*916* and the multiple antibiotic-resistance transposon from *Streptococcus pneumoniae*, Tn*1545* (Courvalin and Carlier, 1986). On the basis of results obtained from studies on both Tn*916* and *Tn1545*, the conjugative transposition process is initiated by the excision of the transposon from DNA of the donor, either the donor chromosome or a resident plasmid. The excision of the transposon is similar to excision of prophage λ in *E. coli* in that staggered cleavages occur at both ends of the transposon (Poyart-Salmeron et al., 1990). These cleavages result in 6-bp protruding 5' hydroxyl ends that differ in sequence (Manganelli et al., 1996; Rudy and Scott, 1996). The transposon then circularizes, followed by ligation of the ends, which results in a 6-bp heteroduplex where the ends were ligated (Caparon and Scott, 1989). If at this point the transposon-containing cell is in contact with a potential recipient, one strand of the ligated element is transferred to the recipient, in which a second, complementary strand is synthesized (Scott et al., 1994). The transposon may then insert into the recipient chromosome or a resident plasmid at a site determined not by any homology to the element, but rather by an AT-rich region in the target DNA, usually shown to contain a static bend (Lu and Churchward, 1995). A 6-bp heteroduplex is formed at each end of the newly integrated transposon via ligation to the site on the element from which it was originally excised from the donor DNA. The mismatched bases at each end of the insertion site are thought to be resolved during normal replication or by mismatch repair (Marra and Scott,

1999). As in intracellular transposition, the only transposon-encoded functions known to be involved in conjugative transposition are Int and Xis.

Antibiotic Resistance

Most enterococcal species are intrinsically resistant to β-lactam antibiotics and to clindamycin, aminoglycosides, and the folate pathway inhibitors, trimethoprim and sulfamethoxazole. Resistance to β-lactams is due to the low affinities of the penicillin-binding proteins produced by *Enterococcus* species (Fontana et al., 1996). Minimum inhibitory concentrations (MICs) of penicillins can range from 2 to greater than 64 µg/ml, depending on specific antibiotic, enterococcal species, or strain. MICs tend to be even higher for the semisynthetic penicillins, and the cephalosporins are clinically ineffective (Murray, 1990). The enterococci are resistant to low levels of aminoglycoside antibiotics, with MICs that may range between 8 and 250 µg/ml, again depending on the aminoglycoside, enterococcal species, or strain, owing to limited transport across the cell membrane. Classically, the intrinsic resistance of this genus to the β-lactam antibiotics and the aminoglycosides has been overcome by the use of antibiotic combinations. The presence of a cell wall inhibitor, such as a β-lactam, appears to increase the uptake of the aminoglycoside by the *Enterococcus*, resulting in a synergistic killing (Moellering and Weinberg, 1971). Acquired high resistance to cell wall inhibitors and to all clinically useful aminoglycosides has eliminated the usefulness of a synergistic approach in the treatment of many enterococcal infections. The enterococci are also intrinsically resistant to the lincosamides, such as lincomycin and clindamycin, with MICs reaching as high as 100 µg/ml (Karchmer et al., 1975; Murray, 1990). The intrinsic resistance of the enterococci to folate pathway inhibitors is due to their ability to utilize exogenous thymidine, thymine, or folates, such as folinic acid, dihydrofolate, and tetrahydrofolate (Hamilton-Miller, 1988).

High Level Aminoglycoside Resistance

One method by which the enterococci resist synergistic killing by a cell wall inhibitor and an aminoglycoside is to acquire genes that encode enzymes that modify one or more aminoglycosides. Three types of aminoglycoside-modifying enzymes that have been described include phosphotransferases (APHs), acetyltransferases (AACs) and nucleotidyltransferases (ANTs), which, respectively, mediate the phosphorylation of a hydroxyl group on the aminoglycoside at the expense of ATP, the acetylation of an amino

group using acetyl coenzyme A as a donor, and the adenylylation of a hydroxyl group with the adenyl group of ATP. Acquisition of these enzymes, which are usually plasmid-encoded, results in high level aminoglycoside resistance, i.e., MICs of the antibiotics affected are greater than 2000 µg/ml and can reach levels greater than 40,000 µg/ml.

High level resistance to streptomycin has been observed in greater than 50% of enterococcal strains isolated as early as the mid-1950s (Jones and Finland, 1957; Atkinson et al., 1997). Two types of streptomycin-modifying enzymes, nucleotidyltransferase, ANT(6)-Ia and ANT(3″)-Ia, encoded by *ant(6)-Ia* and *ant(3″)-Ia*, respectively, have been described (Krogstad et al., 1978; Clark et al., 1999). Mutations in the 30S ribosomal subunit, which result in decreased binding of streptomycin to the ribosome, can also result in streptomycin MICs that may be as high as 128,000 µg/ml (Eliopoulos et al., 1984). One of the most common plasmid-mediated aminoglycoside modifying genes found in enterococci is *aph(3′)-IIIa*, which by encoding the aminoglycoside phosphotransferase, APH(3′)-IIIA, causes high level resistance to kanamycin (Trieu-Cuot and Courvalin, 1983). This gene was identified on kanamycin resistance plasmids harbored by 70, 52 and 79% of enterococci isolated from chickens, pigs, and humans, respectively (LeBlanc et al., 1987). An *ant(9)-Ib* gene, encoding resistance to spectinomycin, via adenylylation at the 9 position, has been isolated from a single *E. faecalis* isolate (LeBlanc et al., 1991).

The most clinically relevant enterococcal gene resulting in aminoglycoside modifying activity is *aac(6′)-Ie-aph(2″)-1a*, which encodes a bifunctional enzyme, AAC(6′)-APH(2″) that mediates resistance to gentamicin, tobramycin, amikacin, kanamycin, netilmicin, and dibekacin (Ferretti et al., 1986; Azucena et al., 1997). This gene was present in the first high-level gentamicin resistant *E. faecalis* isolates described (Horodniceanu et al., 1979), which were also resistant to kanamycin and tobramycin, but not streptomycin, and in the first enterococci to be described as resistant to all aminoglycosides, including gentamicin and streptomycin (Mederski-Samoraj and Murray, 1983; Murray et al., 1983b). Although greater than 90% of gentamicin resistant enterococcal isolates encode the bifunctional enzyme, single aminoglycoside modifying enzymes that mediate resistance to gentamicin, as well as other aminoglycosides, have also been described. For example, *aph(2″)-Ic*, from a strain of *Enterococcus gallinarum*, encodes an aminoglycoside phosphotransferase that results in resistance to gentamicin, tobramycin, kanamycin and dibekacin (Chow et al., 1997). The aminoglycoside phosphotransferase genes, *aph(2″)-Ib*, from a strain of *E. faecium* (Kao et al., 2000), and

aph(2″)-Id, from a strain of *Enterococcus casseliflavus* (Tsai et al., 1998), encode aminoglycoside phosphotransferase activity leading to resistance to gentamicin, tobramycin, kanamycin, netilmicin and dibekacin. The *aph(2″)-Ic* and *aph(2″)-1d* have also been detected in *E. faecium*, and the former has been found in *E. faecalis* as well (Kak and Chow, 2002).

High β-Lactam Resistance

Isolated in 1981, *Enterococcus faecalis* strain HH22 was the first enterococcal strain shown to produce β-lactamase activity (Murray and Mederski-Samoraj, 1983a) and the first high-level gentamicin-resistant *Enterococcus* to be isolated in the United States (Mederski-Samoraj and Murray, 1983). Both the β-lactamase gene and the *aac(6′)-Ie-aph(2″)-1a* gene are encoded by a pheromone-responsive plasmid (Murray et al., 1988). β-Lactamase-producing *E. faecalis* strains have been isolated from patients in at least four countries, many of which have been disseminated by inter- and intra-hospital clonal spread (Murray et al., 1991; Murray et al., 1992b). Even among those isolates that are not clonal, β-lactamase activity is almost always associated with high level gentamicin resistance (Murray, 1992a), and the single β-lactamase producing *E. faecium* isolate described thus far also expresses high level aminoglycoside resistance (Coudron et al., 1992). The gene encoding β-lactamase activity in enterococci was likely acquired from *Staphylococcus aureus* and is nearly identical to the *S. aureus blaZ* gene (Murray, 1992a). Despite the reports describing several isolates in the United States and elsewhere, the incidence of β-lactamase-producing enterococci remains rare (Gordon et al., 1992; Murray, 1998).

High resistance of enterococci to β-lactam antibiotics, particularly penicillins, may also be due to either the overexpression of a low-affinity penicillin-binding protein (PBP) and/or to mutations in a low-affinity PBP structural gene, which lowers its affinity for penicillins to an even greater degree (Fontana et al., 1996). Spontaneous mutants of *E. hirae* strain 9790 (previously designated "*E. faecium* strain 9790") with higher resistance to penicillins were shown to produce higher levels of the low affinity PBP5 (Fontana et al., 1983). Spontaneous mutants of *E. faecalis* and *E. faecium* strains also were shown to produce higher levels of this low affinity PBP. Subsequently, a gene designated "*psr*," located upstream of the PBP5 gene in *E. hirae* 9790 and encoding a 19-kDa protein, was shown to be a negative regulator of PBP5 expression (Ligozzi et al., 1993). Penicillin-resistant mutants had suffered deletions in this gene. Spontaneous mutants of eight different species of *Enterococ-*

cus, selected for resistance to penicillin G, were resistant to all β-lactam antibiotics tested and produced increased levels of a low affinity PBP (al-Obeid et al., 1990). In some clinical isolates of *E. faecalis* against which ampicillin MICs were high (i.e., 32 to 64 µg/ml), higher levels of PBP5 were produced and two other PBPs, 1 and 6, with decreased levels of binding to penicillin were expressed (Cercenado et al., 1996). Penicillin-resistant clinical isolates of *E. faecium* were shown to be resistant via one of the two mechanisms described for the clinical isolates of *E. faecalis* (Zorzi et al., 1996). In these instances, however, strains of *E. faecium* with intermediate levels of resistance (i.e., penicillin MICs between 16 and 64 µg/ml) expressed higher than normal levels of PBP5fm, whereas strains with higher resistance (i.e., MIC = 90 µg/ml) had amino acid substitutions in PBP5fm that resulted in even lower affinities for penicillin.

Recently, a new mechanism of β-lactam resistance was described that involved neither altered expression, binding properties of PBPs, nor production of β-lactamase activity (Mainardi et al., 2000). The resistant mutant strain of *E. faecium* studied contained β-lactam-insensitive L-Lys → D-Asx-L-Lys crosslinks, rather than the normal wild type β-lactam sensitive D-Ala → D-Asn (or D-Asp)-L-Lys crosslinks. Whereas the latter crosslinks are due to PBP-associated DD-transpeptidation activity, the former is due to an LD-transpeptidation that is not associated with any PBP.

Glycopeptide Resistance

The first enterococcal clinical isolate shown to be resistant to glycopeptide antibiotics was a strain of *E. faecium* isolated in 1986, which harbored a plasmid that mediated resistance to both vancomycin and teicoplanin (Leclercq et al., 1988). Subsequently, this resistance was shown to be due to genes encoded on a transposon, Tn*1546*, and the products of these genes was shown to result in the synthesis of a depsipeptide peptidoglycan precursor, rather than the normal pentapeptide ending in D-Ala-D-Ala (Arthur et al., 1993). This original glycopeptide resistance phenotype subsequently became known as "the VanA type," and an additional five phenotypes (VanB to VanE) plus an unclassified type have since been described. The mechanisms of resistance in all types come down to the replacement of D-Ala-D-Ala, the target of glycopeptides, by either D-Ala-D-lactate or D-Ala-D-Ser, either of which has a much lower affinity for glycopeptides than D-Ala-D-Ala. These mechanisms and the genetics of glycopeptide resistance in enterococci have been reviewed recently (Evers et al., 1996; Malathum and Murray, 1999) and will be described only briefly here.

VanA enterococcal isolates express high resistance to both vancomycin and teicoplanin, a phenotype mediated by the *vanA* gene cluster encoded by Tn*1546* (Arthur et al., 1993) or a related transposable element (Handwerger et al., 1995; Palepou et al., 1998; Woodford et al., 1998b). Genetic elements encoding the *vanA* gene cluster have been described in eight different enterococcal species. Tn*1546* and related transposable elements encode nine genes. Two of these, *orf1* and *orf2*, encode proteins related to transposases and resolvases (Arthur et al., 1993). The genes *vanA*, *H*, *X*, *Y* and *Z*, encode proteins directly associated with glycopeptide resistance. VanA is an enzyme that catalyzes the formation of an ester bond between D-Ala and D-lactate (Bugg et al., 1991a; Arthur et al., 1992a). VanH catalyzes the reduction of pyruvate to D-lactate, one of the two substrates of VanA (Bugg et al., 1991b). VanX catalyzes the hydrolysis of D-Ala-D-Ala synthesized by the normal chromosome encoded D-Ala:D-Ala ligase, which helps to insure that D-Ala-D-lactate is incorporated into the pentapeptide (Reynolds et al., 1994a). Some D-Ala-D-Ala synthesized by the chromosomal ligase may still be incorporated into the pentapeptide precursor in VanA strains despite the predominance of D-Ala-D-lactate and the accompanying activity of VanX. However, VanY can catalyze the cleavage of the terminal D-Ala in this pentapeptide, resulting in tetrapeptide-containing precursors that bind less effectively to glycopeptides (Gutmann et al., 1992; Arthur et al., 1994). No enzymatic role has been identified for VanZ, although it may affect resistance of VanA enterococcal strains to teicoplanin (Arthur et al., 1995). The *vanR*/*vanS* genes encode a two-component regulatory system in which VanS recognizes an external signal, likely vancomycin, which results in self-phosphorylation. Then VanR is phosphorylated, activating its own promoter as well as that of the vancomycin resistance structural genes (Arthur et al., 1992b; Silva et al., 1998).

VanB enterococcal isolates express variable levels of resistance to vancomycin but are susceptible to teicoplanin, and resistance is mediated by the *vanB* gene cluster, most often chromosomally located (Malathum and Murray, 1999), but it may also be present on a plasmid (Woodford et al., 1995; Rice et al., 1998b). The *vanB* gene cluster has also been located on the composite transposon, Tn*1547*, within a large chromosomal conjugative element (Quintiliani et al., 1994; Quintiliani and Courvalin, 1996). The VanB phenotype has been associated primarily with strains of *E. faecalis* and *E. faecium* (Malathum and Murray, 1999), and occasionally with *E. casseliflavus* (Woodford, 1998a) and *E. gallinarum* (Liassine et al., 1998). There are six genes in the *vanB* cluster that are homologs of

vanA genes, *vanS_B*, *vanR_B*, *vanH_B*, *vanX_B*, *vanY_B* and *vanB* (Evers and Courvalin, 1996). There is no *vanZ* homolog, but there is an additional gene, *vanW*, of unknown function. The gene cluster is induced by vancomycin, but not by teicoplanin (Evers and Courvalin, 1994). Induction by vancomycin results in resistance to teicoplanin.

VanC resistance is not an acquired trait but rather is intrinsic to *E. gallinarum* and *E. casseliflavus* (Malathum and Murray, 1999). VanC is a D-Ala:D-Ser ligase which results in a pentapeptide precursor with a D-Ala-D-Ser terminal dipeptide (Reynolds et al., 1994b). The VanD phenotype, detected in strains of *E. faecium*, is characterized by a constitutive resistance to both vancomycin and teicoplanin (Perichon et al., 1997; Ostrowsky et al., 1999). It has been chromosomally located only, has not been transmissible, and the pentapeptide, as in VanA strains, ends in D-lactate. The *vanD* gene cluster contains the *vanA*/*vanB* gene cluster homologs (*vanS_D*, *vanR_D*, *vanH_D*, *vanX_D* and *vanY_D*) but no homologs of *vanW* or *vanZ* (Casadewall and Courvalin, 1999). The VanE phenotype, chromosomally determined in a strain of *E. faecalis*, is represented by low resistance to vancomycin and susceptibility to teicoplanin; this phenotype is due to vancomycin-induced synthesis of D-Ala-D-Ser as the terminal dipeptide (Fines et al., 1999).

Resistance to Miscellaneous Antibiotics

Resistance by enterococci to certain antibiotics has been observed in strains isolated as early as the mid-1950s. Greater than 50% of enterococcal isolates obtained from patients in Boston City Hospital between 1953 and 1954 (Jones and Finland, 1957) and in two Washington, DC, area hospitals (Atkinson et al., 1997) were resistant to streptomycin and tetracycline. Whereas 96 and 25% of the Boston isolates were resistant to chloramphenicol and erythromycin, respectively, only 1 and 3%, respectively, of the Washington, DC, isolates were resistant to these two antibiotics. All but 8 of 126 tetracycline-resistant enterococcal isolates from the Washington, DC, area hospitals were also resistant to minocycline, and half of the erythromycin-resistant isolates from these hospitals were also resistant to high levels of lincomycin. None of the strains examined in the latter study (Atkinson et al., 1997) were resistant to ampicillin, gentamicin, or vancomycin, but for 5% of the Boston City Hospital isolates, the MICs of penicillin were >8 µg/ml (Jones and Finland, 1957).

The most common mechanism for resistance to erythromycin among enterococci and Gram-positive bacteria, in general, involves a dimethylation of adenine residues in 23S rRNA within the 50S ribosomal subunit (Horaud et al., 1985; Jensen et al., 1999). These alterations result in the MLS resistance phenotype (Weisblum, 1995), i.e., highly reduced binding to the ribosome by macrolides, such as erythromycin, as well as by newer macrolides (such as azithromycin and clarithromycin; Kak and Chow, 2002), lincosamides (e.g., lincomycin and clindamycin), and streptogramin B antibiotics. MLS resistance among enterococci is almost always encoded by an *erm* (B) gene, which now includes genes previously designated "*erm* (AM)" or "*erm* (AMR)" (Roberts et al., 1999). Occasionally, an MLS-resistant enterococcal isolate will carry an *erm* (A) gene (Roberts et al., 1999; Portillo et al., 2000). MLS-resistance genes are usually plasmid-mediated (Horaud et al., 1985) and may also be located within a transposon, such as Tn*917* (Tomich et al., 1980), or one closely related to it (Banai and LeBlanc, 1984; Rollins et al., 1985). Plasmids encoding MLS resistance often encode one or more additional resistance traits as well (Horaud et al., 1985; LeBlanc et al., 1986).

Among erythromycin resistant isolates obtained from patients in Washington, DC, hospitals in the mid-1950s, half expressed the MLS-resistance phenotype and half were resistant to erythromycin but not lincomycin. While half of the lincomycin resistant isolates expressed the MLS phenotype, the other half was not erythromycin resistant (Atkinson et al., 1997). Those isolates resistant to erythromycin (owing to the expression of an efflux pump) exhibited only the M phenotype and were likely carrying a *mef* (E) gene (Sutcliffe et al., 1996; Tait-Kamradt et al., 1997), currently designated "*mef* (A)" (Clancy et al., 1996; Kak and Chow, 2002). The *mef* (A) gene of many enterococci is located on a large transmissible plasmid (Luna et al., 1999). A *lin* (B) gene (Bozdogan et al., 1999), currently designated "*lnu* (B)" (Roberts et al., 1999), encoding a nucleotidyltransferase that adenylylates both clindamycin and lincomycin, may account for those Washington, DC, enterococcal isolates resistant to lincomycin but not erythromycin.

The reported incidence of chloramphenicol resistance among enterococcal isolates has varied considerably, between <1 and > 50%, dependent to a large extent on the geographical origin, time of isolation or both of the particular strains studied (Jones and Finland, 1957; Toala et al., 1969; Atkinson and Lorian, 1984; Pepper et al., 1986; Atkinson et al., 1997; Jones et al., 2001). Only one mechanism for enterococcal chloramphenicol resistance has been described (i.e., acetylation of a hydroxyl group on chloramphenicol, which inactivates the antibiotic and eliminates ribosomal binding; Shaw, 1983) and is mediated via a plasmid- or chromosome-borne *cat* gene (Pepper et al., 1986; Pepper et al., 1987; Trieu-Cuot et al., 1993).

The incidence of resistance to tetracycline among enterococci appears to vary between 50 and 80%, and as with chloramphenicol resistance, is somewhat dependent on geographical origin and time of isolation (Jones and Finland, 1957; Atkinson and Lorian, 1984; Acar and Buu-Hoi, 1988; Atkinson et al., 1997; Jones et al., 1998). Two mechanisms of tetracycline resistance have been identified in enterococci, active efflux and ribosomal protection (McMurry and Levy, 2000). Determinants encoding these two mechanisms were first classified on the basis of phenotype, i.e., *tet* (L), initially associated with small nonconjugative plasmids, mediated resistance to tetracycline, whereas *tet* (M), usually not found on plasmids, mediated resistance to tetracycline and minocycline (Burdett et al., 1982). Subsequently, TetL was shown to mediate resistance to tetracycline via an energy-dependent efflux mechanism (McMurry et al., 1987). TetM was shown to have no effect on the cellular accumulation of tetracycline, nor did it modify the antibiotic. Rather, cell-free protein biosynthetic machinery from cells containing TetM was resistant to tetracycline (Burdett, 1986), hence TetM was thought to protect ribosomes. From determination of the sequence of *tet* (M) from Tn*916* (Burdett, 1990), TetM protein was found to be homologous to the translation elongation factors EF-G and EF-Tu. Most of the identity between TetM and EF-G lies in the respective GTP-binding domains (Burdett, 1991). Although TetM can protect cellular translation from inhibition by tetracycline, it cannot substitute for either EF-G or EF-Tu (Burdett, 1996).

The *tet* (M) gene is the predominant tetracycline-resistance determinant found in enterococci, and in general, in both Gram-positive and Gram-negative bacteria (Roberts and Hillier, 1990; Roberts, 1997). Other ribosomal protection-mediating genes, such as the *tet* (O) gene (Zilhao et al., 1988; Bentorcha et al., 1991) and the *tet* (S) gene (Charpentier et al., 1994), also have been detected in enterococci. The *tet* (O) gene was originally detected in members of the Gram-negative bacterial genus, *Campylobacter* (Sougakoff et al., 1987), and *tet* (S), originally detected in the Gram-positive bacterial pathogen, *Listeria monocytogenes* (Charpentier et al., 1993). Besides *tet* (L), *tet* (K) is the only other tetracycline-resistance determinant encoding an efflux mechanism detected in enterococci (Roberts, 1997). Although in the enterococci, *tet* (M) is usually chromosome-borne and often on a transposon (many closely related to Tn*916*), it may also be present on a plasmid (Pepper et al., 1987; Zilhao et al., 1988; Bentorcha et al., 1991). The genes, *tet* (L) and *tet* (O), although usually plasmid-borne in enterococci, may also be chromosome-borne (Pepper et al., 1987; Zilhao et al., 1988; Bentorcha et al., 1991), and *tet* (S),

which is plasmid-borne in *Listeria*, was detected in the chromosome of *E. faecalis* (Charpentier et al., 1994). Several resistant enterococcal isolates have been shown to carry more than one tetracycline resistance determinant (LeBlanc and Lee, 1982; Roberts, 1997; Zilhao et al., 1988). A novel tetracycline resistance determinant, *tet* (U) from a strain of *E. faecium*, has been cloned and characterized (Ridenhour et al., 1996). On the basis of its mediation of resistance to both tetracycline and minocycline and its limited homology to *tet* (M) and *tet* (O), a ribosomal protection mechanism was proposed for the gene product Tet(U).

Mechanisms of fluoroquinolone resistance in clinical isolates of *E. faecalis* (Kanematsu et al., 1998) and *E. faecium* (El Amin et al., 1999) have been examined. For both species, high resistance was associated with amino acid substitutions in the quinolone resistance determinant regions (QRDRs) of the A subunit (GyrA) of DNA gyrase and of the C subunit (ParC) of topoisomerase IV. In neither study were the B subunits (GyrB) of DNA gyrase, the E subunits (ParE) of topoisomerase IV, or homologs of known efflux pumps, examined for mutations. Thus, although mutations in *gyrA* and *parC* were clearly associated with high fluoroquinolone resistance in the isolates examined in both studies, additional contributions to resistance of mutations in *gyrB* and *parE*, or in the genes or regulatory elements of efflux systems, cannot be excluded. In the *E. faecalis* study (Kanematsu et al., 1998), 17 isolates exhibiting high resistance had a single amino acid change in the QRDR of GyrA, Ser83Arg, Ser83Ile, Glu87Lys, or Glu87Gly, plus a single change in the QRDR of ParC, Ser80Arg or Ser80Ile. One such isolate had, in addition to a Ser83Ile change in GyrA and a Ser80Ile change in ParC, an additional change in Par C, Glu84Ala. One strain, exhibiting intermediate fluoroquinolone resistance had only a Ser80Arg change in ParC, and no alterations in GyrA. The MICs of ciprofloxacin against 10 isolates in the *E. faecium* study (El Amin et al., 1999) ranged from 32 to >256 µg/ml, and each isolate had a single change in the QRDR of GyrA (Ser83Arg, Ser83Ile, Ser83Tyr or Glu87Lys) and a single change in ParC (Ser80Ile). No specific genotype could be correlated with a specific MIC, leaving open the possibility of mutations in *gyrB*, *parE*, or a gene encoding or regulating an efflux pump.

Linezolid is the only member of a new class of antimicrobial agents, the oxazolidinones, currently in clinical use for the treatment of certain Gram-positive bacterial infections, including infections of vancomycin-resistant enterococci (VRE). The mechanism of action of linezolid is unique in that it inhibits protein synthesis by interfering with the complexing of 70S ribo-

somes, mRNA, IF2, IF3, and fmet-tRNA (Swaney et al., 1998). Although the results of in vitro studies had indicated that the development of resistance to linezolid occurred at a very low frequency, linezolid-resistant VRE were isolated from patients shortly after its introduction into clinical practice. Two of these isolates, both strains of *E. faecium*, were each shown to have undergone a G2576U conversion in the peptidyl transferase region of 23S rRNA (Zurenko et al., 1999). The patients from whom the resistant strains were isolated had received prolonged courses of linezolid for VRE bacteremia. Subsequently, a collection of clinical VRE isolates susceptible to linezolid were subjected to serial passages in doubling concentrations of the antibiotic, and the emergence of resistance was monitored (Prystowsky et al., 2001). *Enterococcus faecalis* isolates began to develop resistance in three passages. After the final passages, the MICs of linezolid ranged from 16 to 128 µg/ml, whereas resistant *E. faecium* strains did not begin to develop their resistance until the tenth passage, and the linezolid MICs reached were only between 8 and 16 µg/ml. The resistant *E. faecalis* mutants were shown to have suffered the same G2576U conversions as the previously studied resistant *E. faecium* clinical isolates (Zurenko et al., 1999), whereas the single in vitro-derived resistant *E. faecium* mutant from which 23S rRNA was examined had a single mutation, G2505A. A recent study has confirmed the role of a G2576U conversion in 23S rRNA in the resistance to linezolid of clinical isolates of *E. faecium* and one *E. faecalis* isolate (Marshall et al., 2002). However, the results of this latter study also showed that the level of resistance to linezolid could be correlated with the number of 23S rRNA genes that contained the mutation, G2576T. The number of 23S rRNA genes with the G2576T mutation and the corresponding MICs of linezolid against the *E. faecium* isolates examined were 0/6 = 2 µg/ml, 1/6 = 8 µg/ml, 2/6–3/6 = 32 µg/ml, and 4/6–5/6 = 64 µg/ml. For the single *E. faecalis* isolate examined, 4/4 23S rRNA genes had the mutation, and the linezolid MIC was 128 µg/ml.

Plasmid-mediated Virulence Traits

Two plasmid-mediated traits, aggregation substance (AS; reviewed in Muscholl-Silberhorn et al., 2000) and cytolysin (reviewed in Haas and Gilmore [1999] and Gilmore et al. [2002]), have been linked to the virulence of *E. faecalis*. AS is encoded by all pheromone-responsive plasmids, and cytolysin may be encoded by pheromone-responsive and non-pheromone-responsive plasmids. A role of AS in host cell adherence was predicted on the basis of the presence of two Arg-Gly-Asp (RGD) motifs in the AS sequence (Galli et al., 1990). Subsequently, a strain of *E. faecalis* that expressed AS constitutively was shown to bind to pig renal tubular cells considerably better than a plasmid-free strain or a plasmid-containing strain in which AS expression was uninduced (Kreft et al., 1992). The adherence could be blocked by the addition of synthetic RGD peptide. The expression of AS has also been correlated with the uptake of *E. faecalis* by intestinal epithelial cells (Olmsted et al., 1994), and the ability of AS to bind host integrins has been associated with the interaction of these bacteria with polymorphonuclear neutrophils (PMNs; Vanek et al., 1999) and with macrophages (Sussmuth et al., 2000), which in turn stimulates enterococcal phagocytosis by these host cells. Survival of enterococci in PMNs and macrophages apparently is enhanced by AS-inhibited phagolysosome acidification (Rakita et al., 1999) and respiratory burst (Sussmuth et al., 2000), respectively. Although AS has been shown to lead to increased vegetation size in a rabbit endocarditis model (Chow et al., 1993; Schlievert et al., 1998), no such effect was observed in a rat endocarditis model (Berti et al., 1998).

The cytolysins produced by *E. faecalis* are able to lyse a variety of eukaryote cell types, including erythrocytes, as well as several species of Gram-positive bacteria (Haas and Gilmore, 1999). Thus these molecules have been designated "bacteriocins" as well as "cytolysins." The cytolysins have been shown to enhance virulence in a variety of infection models (Gilmore et al., 2002). The cytolysin operon, as well as its regulation, has received considerable attention (Haas and Gilmore, 1999; Gilmore et al., 2002). Briefly, the cytolysin molecule is comprised of two peptides, $CylL_L$ and $CylL_S$, which are subjected to post-translational cytoplasmic modification by CylM. The modified products, Cyl_L* and Cyl_S*, are then secreted from the cell by the ATP-binding transporter, CylB, with the secretion of Cyl_L*, but not Cyl_S*, dependent on ATP hydrolysis. Leader sequences on both, Cyl_L* and Cyl_S* are removed during secretion by a CylB-associated serine protease activity. The extracellular products (Cyl_L' and Cyl_S') are again cleaved by another serine protease, CylA, which is also extracellular, yielding the active cytolysin subunits, Cyl_L" and Cyl_S". The regulation of cytotoxin expression has been partially elucidated (Haas et al., 2002). Synthesis of the cytolysin appears to be dependent on cell density, with $CylL_S$" serving as an autoinduction signal. By a mechanism yet to be elucidated, the products of two additional genes (*cylR1* and *cylR2*) apparently function as represssors of the operon in the absence of $CylL_S$".

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CHAPTER 1.2.7

The Genus Lactococcus

MICHAEL TEUBER AND ARNOLD GEIS

Introduction

The importance of lactococci in the development of basic microbiology, genetics, molecular biology, general microbial biochemistry, food science, and biotechnology has been profound. Their present-day large-scale use in industrial fermentations, especially in the manufacture of dairy products has particular significance.

The first studies of the lactococci were by Joseph Lister (Lister, 1873), who was attempting to prove Pasteur's germ theory of fermentative changes. In Lister's experiments with boiled milk as a nutrient medium, he obtained by chance the first pure bacterial culture. It is worthwhile to recall in the context of this handbook his original discussion of this discovery, marking the dawn of bacterial taxonomy:

Admitting then that we had here to deal with only one bacterium, it presents such peculiarities both morphologically and physiologically as to justify us, I think, in regarding it a definite and recognizable species for which I venture to suggest the name *Bacterium lactis*. This I do with diffidence, believing that up to this time no bacterium has been defined by reliable characters. Whether this is the only bacterium that can occasion the lactic acid fermentation, I am not prepared to say.

Taxonomy

This bacterium was later renamed "*Streptococcus lactis*" (Löhnis, 1909; Orla-Jensen, 1919). On the basis of exhaustive reinvestigations, Schleifer et al. (1985) proposed that the N streptococci (Lancefield, 1933) be separated from the oral streptococci, enterococci and hemolytic streptococci, and suggested the new genus name "*Lactococcus*." The position of the lactococci within the clostridium branch of the evolutionary phylogenetic tree is shown in Fig. 1. The lactococci are clearly separated from pathogenic genera of streptococci (Stackebrandt and Teuber, 1988).

The molecular taxonomy of the lactococci has yielded the detection and differentiation of new species besides *L. lactis*: *L. garviae*, *L. raffinolactis*, *L. plantarum* and *L. piscium*. *Lactococcus garviae* has been identified as a causative agent of bovine mastitis and fish lactococcosis (Eldar et al. 1999; Vela et al., 2000). In rare instances, *L. lactis* has been isolated from human cases of urinary tract and wound infections, and from patients with endocarditis (Aguirre and Collins, 1993). In this behavior, it resembles other lactic acid bacteria.

Patrick Tailliez (Tailliez, 2001), on the basis of his interesting study of the phylogeny of lactic acid bacteria including lactococci, has proposed that the lactic acid bacteria appeared before the photosynthetic cyanobacteria. As these were found in sediments dated 2.75 billion years old, lactic acid bacteria may have emerged 3 billion years ago before the atmosphere contained appreciable amounts of oxygen—a proposal that is also suggested by their poor adaptation to aerobic environments. On the other hand, lactococci are clearly on their way to having respiration (Duwat et al., 2001).

The purpose of this chapter is to outline the new developments as well as provide the basic facts about the habitats, enrichment, isolation, and differentiation of the lactococci.

Habitats

The most important habitats of lactococci (Tables 1 and 2) are found in the various niches of the dairy industry environment.

The lactococci comprise the species *Lactococcus lactis*, *L. garviae*, *L. plantarum*, *L. piscium* and *L. raffinolactis* (Table 3).

Lactococcus lactis subsp. *lactis* and *L. lactis* biovar "*diacetylactis*" (according to Schleifer et al., 1985, the subspecific status *L. lactis* subsp. "*diacetylactis*" is no longer valid because of the importance of diacetyl-forming lactococci, especially in dairy fermentations where the distinction between *L. lactis* subsp. *lactis* and *L. lactis* biovar "*diacetylactis*" is more practical and preferred) have commonly been detected directly or following enrichment in plant material, including

fresh and frozen corn, corn silks, navy beans, cabbage, lettuce, peas, wheat middlings, grass, clover, potatoes, cucumbers, and cantaloupe (Sandine et al., 1972). Lactococci are usually not found in fecal material or soil. *Lactococcus lactis* MG1363 did not survive ingestion by human volunteers and disappeared within 4 hours from their ileal fluids (Vesa et al., 2000). Environmental screening revealed only small numbers on the outer surface and in the saliva of cows. *Lactococcus lactis* and its subspecies *lactis* and *cremoris* were detected without enrichment in raw milk, milking machines, and the udders at 10^3–10^4 colony forming units (CFU) per gram or cm^2, respectively, and after enrichment, in the saliva, on the skin of cows and bulls, on grass, and in soil and silage (Klijn et al., 1995). In a dairy environment, lactococci were abundant in cheese milk, cheese whey, waste whey, and the wastewater tank. After enrichment, *L. lactis* was also found in the wastewater disposal site soil and on grass. *Lactococcus garviae* and *L. raffinolactis* were also consistently detected in raw milk, on the skin and in saliva of cows, and on grass (Table 4). Since raw cow's milk consistently contains *L. lac-*

tis subsp. *lactis*, *L. lactis* biovar "*diacetylactis*" and *L. lactis* subsp. *cremoris*, it is tempting to suggest that lactococci enter the milk from the exterior of the udder during milking and from the feed, which may be the primary source of inoculation. The main habitats of *Lactococcus lactis* subsp. *cremoris* seems to be milk, fermented milk, cheese, and starter cultures.

A study of lactococci isolated from animal material in Belgium revealed the following habitats (Pot et al., 1996):

Lactococcus lactis subsp. *lactis*: bovine intestine, milk from healthy cows and cows with mastitis, and tonsils of cats, dogs and goats.

Lactococcus garviae: bovine milk and tonsils, tonsils of dogs, feces of cats and horses, and conjunctiva of a turtle.

Lactococcus raffinolactis: bovine tonsils and goat intestine.

Lactococcus piscium was consistently detected in vacuum packed, refrigerated beef in Japan, when incubation of the plated samples was at 7°C for 10–14 days (Sakala et al., 2002). The type strain of *L. piscium* was isolated from a diseased rainbow trout (Williams et al., 1990).
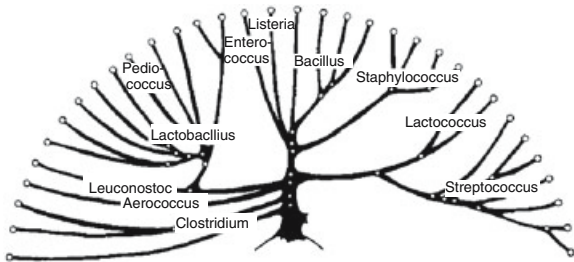
Fig. 1. Evolutionary tree of the *Clostridium* branch of Gram-positive bacteria showing the position of *Lactococcus lactis* and *L. raffinolactis* as an offshoot of the *Streptococcus* branch. The data are based on 16S rRNA oligonucleotide sequence similarities. (From Stackebrandt and Teuber, 1988.)

Table 2. World production of milk, cheese, butter and other dairy products in 2001. Although not all cheese varieties are made with lactococci as starter cultures (notable exception: Swiss-type cheeses), the main varieties like Gouda, Edamer, Cheddar, Camembert, Brie etc. are produced with lactococci. Also, sour cream and sour milk need lactococci (for details see Teuber, 2000; FAO, 2001).

1. Cow milk, whole fresh	490,785,938 Mt*
2. Cheeses (all kinds)	16,260,926 Mt
3. Butter and Ghee	7,064,949 Mt
4. dry whey	1,848,057 Mt
5. dry whole cow milk	2,565,412 Mt
6. evaporated and condensed milk	3,806,105 Mt

*Mt = Metric tons.

Table 1. *Lactococci* as components of starter cultures for fermented dairy products.[a]

Type of product	Composition of starter culture
1. Cheese type without eye formation (Cheddar, Camembert, Tilsit)	*Lactococcus lactis* subsp. *cremoris*, 95 to 98%; *Lactococcus lactis* subsp. *lactis*, 2 to 5%
2. Cottage cheese, quarg, fermented milks, cheese types with few or small eyes (e.g., Edam)	*Lactococcus lactis* subsp. *cremoris*, 95%; *Leuconostoc mesenteroides* subsp. *cremoris*, 5%; or *Lactococcus lactis* subsp. *cremoris*, 85 to 90%; *Lactococcus lactis* subsp. *lactis*, 3%; *Leuconostoc mesenteroides* subsp. *cremoris*, 5%
3. Cultured butter, fermented milk, buttermilk, cheese types with round eyes (e.g., Gouda)	*Lactococcus lactis* subsp. *cremoris*, 70 to 75%; *Lactococcus lactis* subsp. "*diacetylactis*," 15 to 20%; *Leuconostoc mesenteroides* subsp. *cremoris*, 2 to 5%
4. Taette (Scandinavian ropy milk)	*Lactococcus lactis* subsp. *cremoris* (ropy strain)
5. Viili (Finnish ropy milk)	*Oidium lactis* (covers surface); *Lactococcus lactis* subsp. *cremoris* (ropy strain)
6. Casein	*Lactococcus lactis* subsp. *cremoris*
7. Kefir	Kefir grains containing lactose-fermenting yeasts (e.g., *Candida kefir*), *Lactobacillus kefir, Lactobacillus kefiranofacians, Lactococcus lactis* subsp. *lactis*

[a]The quantitative composition has been taken from the culture catalog of a major worldwide supplier.

Table 3. Characteristics differentiating species and subspecies of the genus Lactococcus.

Species and subspecies	Source	Peptidoglycan type*	Major menaquinones	Acid production from							Hydrolysis of arginine
				Galactose	Lactose	Maltose	Melibiose	Melizitose	Raffinose	Ribose	
L. lactis subsp. lactis	Raw milk and dairy products	Lys-D-Asp	MK-9,MK-8	+	+	+	–	–	–	+	+
L. lactis subsp. *cremoris*	Raw milk and dairy products	Lys-D-Asp	MK-9,MK-8	+	+	–	–	–	–	–	–
L. lactis subsp. *hordniae*	Leaf hopper	Lys-D-Asp	MK-8,MK-9	–	–	–	–	–	–	–	+
L. garviae	Bovine samples, diseased fish	Lys-Ala-Gly-Ala	MK-9,MK-8	+	+	V	V	–	–	+	+
L. plantarum	Frozen peas	Lys-Ser-Ala	–	–	–	+	–+	+	–	–	–
L. raffinolactis	Raw milk	Lys-Thr-Ala	–	+	+	+	+	V	+	V	V
L. piscium	Vacuum-packed beef, diseased fish	n.d.	n.d.	+	+	+	+	+	+	–	–

Abbreviations according to Schleifer and Kandler (1972). Asp. = aspartic acid; gly = glycine; lys = lysine, ser = serine; thre = threonine.
Abbreviations according to Collins and Jones (1981). MK-8 = menaquinone with n = 8 isoprene units; MK-9 = menaquinone with n = 9 isoprene units.
+ = positive, – = negative, V = variable, n.d. = not determined.
Adapted from Schleifer (1987) and Sakala et al. (2001).

Table 4. *Lactococcus* species isolated during environmental screening (modified from Klijn et al. 1995).

Sample	*L. lactis lactis*	*L. lactis cremoris*	*L. garviae*	*L. raffinolactis*	New, undefined *Lactococcus* sp.
Cheese plant					
– cheese milk	+	+			
– cheese whey	+	+			
– waste whey	+	+	(+)		
– wastewater tank	+	+	+	+	+
– wastewater disposal site soil	(+)	(+)		(+)	
– grass	(+)	(+)	(+)	(+)	
Farm samples					
– raw milk	+	+	+	+	+
– milk machine	+	+			
– udder	+	+			
– Saliva, cow	(+)	(+)			
– Saliva, bull	(+)				
– skin, cow	(+)		(+)		
– skin, bull	(+)		(+)		
– grass		(+)		(+)	
– soil	(+)		(+)	(+)	
– silage	(+)				

+ = detected by direct plating; (+) = detected by plating after enrichment.

The results of a dairy environmental screening have shown that new, not yet differentiated *Lactococcus* species do exist in nature if modern molecular methods such as 16S RNA-gene sequencing are employed (Klijn et al., 1995). Another interesting habitat seems to be the microflora of the gut of wood-feeding termites such as *Reticulitermes flaviceps*. The Gram-positive cocci (comprising about 50% of the culturable microflora) contained new species closely related to *Enterococcus faecalis*. Three other studied strains cluster closely with *L. garviae*, but definitely represent a new species (Bauer et al., 2000).

In summary, animal environments are the main habitats of the investigated lactococci. The extent to which lactococci used by the dairy industry have undergone evolutionary changes as a result of their deliberate use as starter cultures remains to be established. Their original habitat is and was the dairy cow and the raw milk extracted from it. Recently *L. lactis* subsp. *cremoris* was re-isolated from samples of Chinese and Moroccan raw milk, and one corn (*Zea mays*) sample, but not from *Rubus discolor* (Himalayan blackberry), *Hypericum calycinum* (gold flower), *Prunus* sp. (plum), *Hedera helix* (English ivy), *Acer platanoides* (green lace tree), and *Gingko biloba* (maidenhair tree; Salama et al., 1993).

Enrichment and Isolation

Lactic acid bacteria are nutritionally fastidious. They all require complex media for optimal growth. In synthetic media, all strains of lactococci require amino acids such as isoleucine, valine, leucine, histidine, methionine, arginine, and proline, and vitamins (niacin, Ca-pantothenate, and biotin; Anderson and Elliker, 1953; Jensen and Hammer, 1993).

Isolation from Plant Material

Plant material, e.g., grass and herbages, is the natural source of lactic acid bacteria. Ensilage allows the enrichment of lactococci, leuconostocs and pediococci. The isolation procedure takes advantage of the sequential growth of the above mentioned genera (Whittenbury, 1965).

Grasses and other plant material are collected and cut in pieces as aseptically as possible. The prepared material is then placed in sterile glass tubes and compressed. Fifty grams of material are enough to fill a 3×20 cm tube. The tubes are sealed in a way that permits pressurized gas to escape but prevents the entry of oxygen. A number of silages are prepared and incubated at 30°C. Tubes are opened beginning on the second day.

The silage is removed and placed in flasks of sterile water, which are vigorously shaken. These suspensions can then either be streaked directly onto agar plates or diluted and pour plated. The 2- to 3-day-old silages are the best sources for lactococci and leuconostocs; 4–7-day-old silages are best for pediococci.

A modification of the above method has been reported by Weiler and Radler (1970). Grape leaves are homogenized with the same amount of acetate buffer (pH 5.4; 0.2 M). The homoge-

nate is placed in sterile tubes. The tubes are sealed and incubated at 30°C.

Isolation from Dairy Products

The main problem in isolating lactic acid bacteria from dairy products is a proper dissolution or dispersion of the solid or semisolid fat-containing material.

ISOLATION OF LACTIC ACID BACTERIA FROM CHEESE OTHER THAN COTTAGE CHEESE The procedure is described below and in Olson et al. (1978).

Using aseptic technique, thoroughly comminute or mix each sample until representative portions can be removed. Heat 99-ml dilution blanks of sterile, freshly prepared (less than 7 days old), aqueous 2% sodium citrate to 40°C. Aseptically transfer 11 g of cheese to a sterile blender container previously warmed to 40°C and add the warmed sodium citrate blank. Mix for 2 min at a speed sufficient to emulsify the sample properly, invert the container to rinse particles from the interior walls, and remix for approximately 10 s. Inadvertent heating to temperatures in excess of 40°C from friction in agitation may occur with some mechanical blenders. This should be determined before use of the blender so corrective action can be taken, if needed. If heating is unavoidable with equipment available, mixing periods of less than 2 min should be used provided that complete emulsification is obtained. A 1 : 10 dilution should be plated or further diluted immediately, great care being taken to avoid air bubbles or foam.

As an alternative method, 1 g ± 10 mg is rapidly weighed into a presterilized 177-ml (6 oz) Whirl-Pak bag or its equivalent. Close the bag, transfer it to a flat surface, and macerate the contents into a fine paste by rolling a 15 × 125 mm test tube or similar cylindrical object over the bag. The sample should not be forced into the corners nor the ties allowed to seal off areas of the bag. Open the bag and add 9 ml of 2% sodium citrate at 40°C. Reclose the bag and roll the contents, as described above, to form a fine emulsion and proceed with plating immediately. Enumeration of bacterial species that form chains, such as lactic streptococci, may not be feasible with this method. However, this problem was not evident in a collaborative study involving analysis of Cheddar and Romano cheeses in nine laboratories in the United States.

ISOLATION OF LACTIC ACID BACTERIA FROM COTTAGE CHEESE The procedure is described below and in Olson et al. (1978).

Place the sterile blender container on a proper balance and tare. With a sterile spatula, mix contents of the cottage cheese container; or if in a tightly closed plastic sample pouch, gently knead and mix the enclosed curd.

Aseptically remove the cover of the blender and place it upside down on the balance beside the container. Weigh 11 g of cottage cheese into the sterile container. Add 99 ml of warmed (40°C) sterile 2% sodium citrate solution as described above (see Isolation from Cheese Other than Cottage Cheese) to disperse and dilute the cottage cheese curd. Proceed as above and plate appropriate dilutions immediately. The alternative bag method (described in Isolation from Cheese Other than Cottage Cheese) may also be used.

ISOLATION OF LACTIC ACID BACTERIA FROM CULTURED MILK, CULTURED CREAM, YOGURT, ACIDOPHILUS MILK, BULGARIAN BUTTERMILK, AND SIMILAR CULTURED OR ACIDIFIED SEMIFLUID PRODUCTS: THE PROCEDURE IS DESCRIBED BELOW AND IN OLSON ET AL. (1978) After thoroughly mixing the sample, weigh 11 g of product into a sterile widemouth container, add 99 ml of sterile buffered distilled water (40°C), shake until a homogeneous dispersion is obtained, and withdraw appropriate amounts of this 1 : 10 dilution for plating or further dilution. The sample may be dispersed in 2% sodium citrate with a mechanical blender as described above (see Isolation from Cottage Cheese).

Unfortunately, no satisfactory selective medium is available for the isolation of lactococci. Two media, both commercially available, are generally accepted to give reliable growth of these organisms. The medium proposed by Elliker et al. (1956) is widely used for the isolation and enumeration of lactococci. Ml7 medium (Terzaghi and Sandine, 1975), a complex medium supplemented by 1.9% β-disodium glycerophosphate, resulted in improved growth of lactococci.

Elliker Agar Medium for Isolation of Lactococci
(Elliker et al., 1956)

Tryptone	20.0 g
Yeast extract	5.0 g
Gelatin	2.5 g
Dextrose	5.0 g
Lactose	5.0 g
Sucrose	5.0 g
Sodium chloride	4.0 g
Sodium acetate	1.5 g
Ascorbic acid	0.5 g
Agar	15.0 g

The ingredients are dissolved in and diluted up to 1 liter with distilled water. The medium has a pH of 6.8 before autoclaving.

This medium is probably the most cited for the isolation and growth of lactococci, although it is unbuffered. This disadvantage can be overcome by the addition of suitable buffer substances. Addition of 0.4% (w/v) of diammonium phosphate improves the enumeration of lactic streptococci on Elliker agar. Colony counts were up to about eight times greater owing to improved buffering capacity (Barach, 1979).

M17 Medium for Isolation of Lactococci (Terzaghi and Sandine, 1975)

Phytone peptone	5.0 g
Polypeptone	5.0 g
Yeast extract	5.0 g
Beef extract	2.5 g
Lactose	5.0 g
Ascorbic acid	0.5 g
β-Disodium glycerophosphate	19 g
$MgSO_4 \cdot 7H_2O$ (1.0 M)	1.0 ml
Glass-distilled water	1 liter

The medium is sterilized at 121°C for 15 min. The pH of the broth is 7.1. Solid medium contains 10 g of agar/liter of medium.

This medium is useful for the isolation of all strains of *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis*, *L. lactis* biovar "*diacetylactis*," and *Streptococcus thermophilus* and mutants of those strains lacking the ability to ferment lactose.

Addition of a pH indicator dye (bromcresol purple) and reduction of β-disodium glycerophosphate (5 g/liter) allows an easy differentiation between lactose-fermenting (large yellow colonies) and nonfermenting (small white colonies) strains (Kondo and McKay, 1984).

Shankar and Davies (1977) demonstrated the suppression of *Lactobacillus delbrueckii* subsp. *bulgaricus* in M17 medium. The majority of these *Lactobacillus* strains failed to grow in this medium adjusted to pH 6.8. Since M17 medium supported good growth of *Streptococcus thermophilus*, it can be used for the selective isolation of this microorganism from yogurt.

In recent years, this medium has become the standard for genetic investigations of lactococci (see below).

Lactococcal bacteriophages can be efficiently demonstrated and distinguished on M17 agar. Plaques larger than 6 mm in diameter could be observed as well as turbid plaques, indicating lysogeny (Terzaghi and Sandine, 1975).

Enumeration of Citrate-Fermenting Bacteria in Lactic Starter Cultures and Dairy Products

To control gas and aroma (diacetyl) production in the fermentation of various dairy products, it is important to know the quantitative composition of the used starter cultures.

Leuconostoc species and *Lactococcus lactis* biovar "*diacetylactis*" are components of many mesophilic starter cultures (Table 1). These organisms are able to ferment citrate with concomitant production of CO_2 and diacetyl.

For the collective enumeration of leuconostocs and *L. lactis* biovar "*diacetylactis*" in starters and fermented dairy products, a whey agar containing calcium lactate and casamino acids (WACCA) has been introduced by Galesloot et al. (1961).

WACCA 0.5% Medium for Enumeration of *Leuconostoc* and *Lactococcus lactis* biovar "*diacetylactis*" (Galesloot et al., 1961).

Composition of the medium:

Dissolve in 1 liter of whey, 5 g of Ca lactate ·5 H_2O, 7 g of casitone, and 0.5% yeast extract. Adjust to pH 7.3 with $Ca(OH)_2$ suspension. Steam for 30 min, filter and adjust to pH 7.1 with a NaOH solution. Add 1 ml of $MnSO_4$ solution (40 mg of $MnSO_4 \cdot 4 H_2O$/100 ml). Dissolve 15 g of agar, clarify with 5 g of albumin, and sterilize for 15 min at 110°C (15 ml/tube).

Preparation of whey:

Add to 1 liter of high-temperature-short-time (HTST)-pasteurized fresh skim milk at 30°C, 0.3 ml of 35% $CaCl_2$ solution and 0.3 ml of commercial rennet (strength 1 : 10,800). Cut coagulum after 30 min at 30°C. Filter after 2 h at 45°C.

Preparation of Ca-citrate suspension:

Suspend 28 g of Ca citrate (Merck) in a 100 ml of 1.5% carboxymethyl cellulose solution prepared at 45°C. Allow to precipitate for 2 h at 45°C. The supernatant is steamed for 30 min.

Application:

Add 0.3–0.7 ml of Ca citrate suspension per 15-ml of WACCA (0.5%; 48°C). The amount to be added has to be adapted to the type of starter under investigation. Incubate for 5 days at 25°C. Count after 2, 3 and 5 days.

A different medium developed by Nickels and Leesment (1964) for the same purpose gives comparable results. A modified medium based on the different action of the lactose analogue 5-bromo-4-chloro-3-indolyl-*p*-D-galactopyranoside (Xgal) has been suggested (Vogensen et al., 1987).

Identification

Lactococci are Gram-positive, microaerophilic cocci which were thought to lack the cytochromes of the respiratory chain (Hardie, 1986). However, the complete genome sequence obtained from *L. lactis* subsp. *lactis* IL1403 (Bolotin et al., 2001) showed the presence of a complete cytochrome *d* oxidase (subunits I and II: *cydA* and *cytB*). If heme is present in the medium, this leads to respiration under certain

conditions, for example, during late exponential growth phase (Duwat et al., 2001).

Lactococci can be simply differentiated from pediococci and leuconostocs by the main fermentation products from glucose (see Table 5).

The common morphology consists of spherical or ovoid cells, 0.5–1 µm in diameter, in pairs or more or less long chains (Figs. 2–5). The dairy lactic streptococci are in practice differentiated

by their growth behavior at different temperatures (Table 6) into the mesophilic species *Lactococcus lactis* (Schleifer et al., 1985) and the thermophilic species *Streptococcus thermophilus*. *Streptococcus thermophilus* is differentiated at first glance from the enterococci by its inability to grow in the presence of 6.5% NaCl. Problems
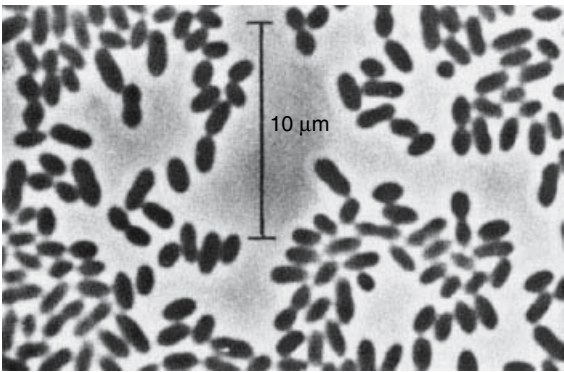
Table 5. Differentiation scheme for lactococci, pediococci, and leuconostocs.

Fermentation products of glucose	Genus
L(+)-Lactic acid	*Lactococcus*
D(−)-Lactic acid, CO_2, acetic acid, ethanol	*Leuconostoc*
DL-Lactic acid	*Pediococcus*

Fig. 3. Phrase-contrast micrograph of *Lactococcus lactis* subsp. *lactis*. This strain is forming pairs of ovoid cells.

Fig. 4. Phase-contrast micrograph of *Lactococcus lactis* subsp. *lactis*. This strain is forming chains. Bar = 10 µm.

Fig. 2. Electron micrograph of thin sections of *Lactotoccus lactis* subsp. *lactis* at an early stage after infection with a specific bacteriophage. The large cells show the typical features of a Gram-positive bacterium. It clearly demonstrates the almost rodlike appearance of a cell just starting to divide (compare to Figs. 3, 4, and 5). (Courtesy of J. Lembke.)
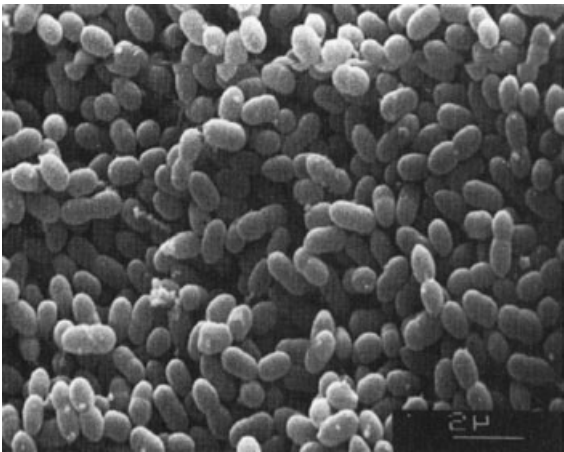
Fig. 5. Scanning electron micrograph of *Lactococcus lactis* subsp. "*diacetylactis*" growing in pairs of ovoid cells. Bar = 2 µm.

Table 6. Physiological and other properties of dairy lactococci used for identification and differentiation.

Properties	*L. lactis* subsp. *lactis*	*L. lactis* subsp. "*diacetylactis*"	*L. lactis* subsp. *cremoris*
Growth at 10°C	+	+	+
Growth at 40°C	+	+	−
Growth at 45°C	−	−	−
Growth in 4% NaCl	+	+	−
Growth in 6.5% NaCl	−	−	−
Growth at pH 9.2	+	+	−
Growth with methylene blue (0.1% milk)	+	+	−
Growth in presence of bile (40%)	+	+	+
NH_3 from arginine	+	+	−
CO_2 from citrate	−	+	−
Diacetyl and acetoin	−	+	−
Fermentation of maltose	+	+	Rarely
Hydrolysis of starch	−	−	−
Heat resistance (30min at 60°C)	V	V	V
Serological group[a]	N	N	N
GC content of DNA[b] (mol%)	33.8–36.9	33.6–34.8	35.0–36.2

+, positive; −, negative; V, variable.
[a]Lancefield, 1933.
[b]Knittel, 1965.

may arise in the identification of *L. lactis* biovar "*diacetylactis*" if the plasmid-coded fermentation of citrate is lost. The G+C content of DNA ranges from 34 to 43 mol% (Schleifer et al., 1985). The genome sizes of different lactococci were estimated to be 2,300–2,600 kb (Le Bourgeois et al., 1989).

Since the mesophilic dairy species differ only in a few properties (Table 6), the G+C data are meaningless for this differentiation. That the three mesophilic dairy species are closely related is also implicated by the observation that many bacteriophages cross the "subspecies" line and attack strains of all three species (Lembke et al., 1980). Also, the plasmid patterns investigated so far do not allow a species differentiation (Fig. 6), but do allow the reidentification of strains. Another very useful and efficient approach is differentiation on the basis of protein patterns after gel electrophoresis of cell extracts (Jarvis and Wolff, 1979; Pot et al., 1996; Sakala et al., 2002). By this method, classification of closely related strains and assignments to species and subspecies is possible if proper comparison with known strains is included.

Collins and Jones (1979) have shown that the lactococci contain menaquinones with nine isoprene units as the major component, in contrast to dimethylmenaquinones with nine isoprene units and menaquinones with eight isoprene units in enterococci. At the moment, it is not possible to assess the number of different lactococci strains existing in dairies and starter cultures throughout the world. However, *L. lactis* subsp. *cremoris* strains seem to be scarce (Salama et al., 1993).

Molecular approaches include 16S and 23S rRNA-targeted oligonucleotide probes (Betzl et al., 1990; Salama et al., 1991; Salama et al., 1993; Beimfohr et al., 1993), and corresponding polymerase chain reaction (PCR)-based methods (Deasy et al., 2000). A well established method to draw a phylogenetic tree of all lactococcal species is based on the complete nucleotide sequences of their 16S rRNA-genes (Teuber, 1995; see Fig. 7).

Applications

The species *Lactococcus lactis* and its subspecies used on a large scale by the dairy industry are generally recognized as safe (GRAS) for human consumption. Their deliberate use in the dairy industry as starter cultures for many different products is documented in Tables 1 and 2. This development was initiated at the turn of the twentieth century when H. Weigmann, V. Storch, and H. W. Conn identified lactococci as the essential components of the mesophilic microflora in spontaneously fermented cream and milk. This finding led to the introduction of pure starter cultures of lactic acid bacteria to the dairy field for use in the fermentation and ripening of milk, cream, and cheese (Weigmann, 1905–1908).

In recent years, the physiology, biochemistry, genetics, and molecular biology of the lactococci have gained much attention, owing to the great economic importance of these organisms (Teuber, 1995).

Lactococci are employed in single and mixed cultures for the production of different kinds of
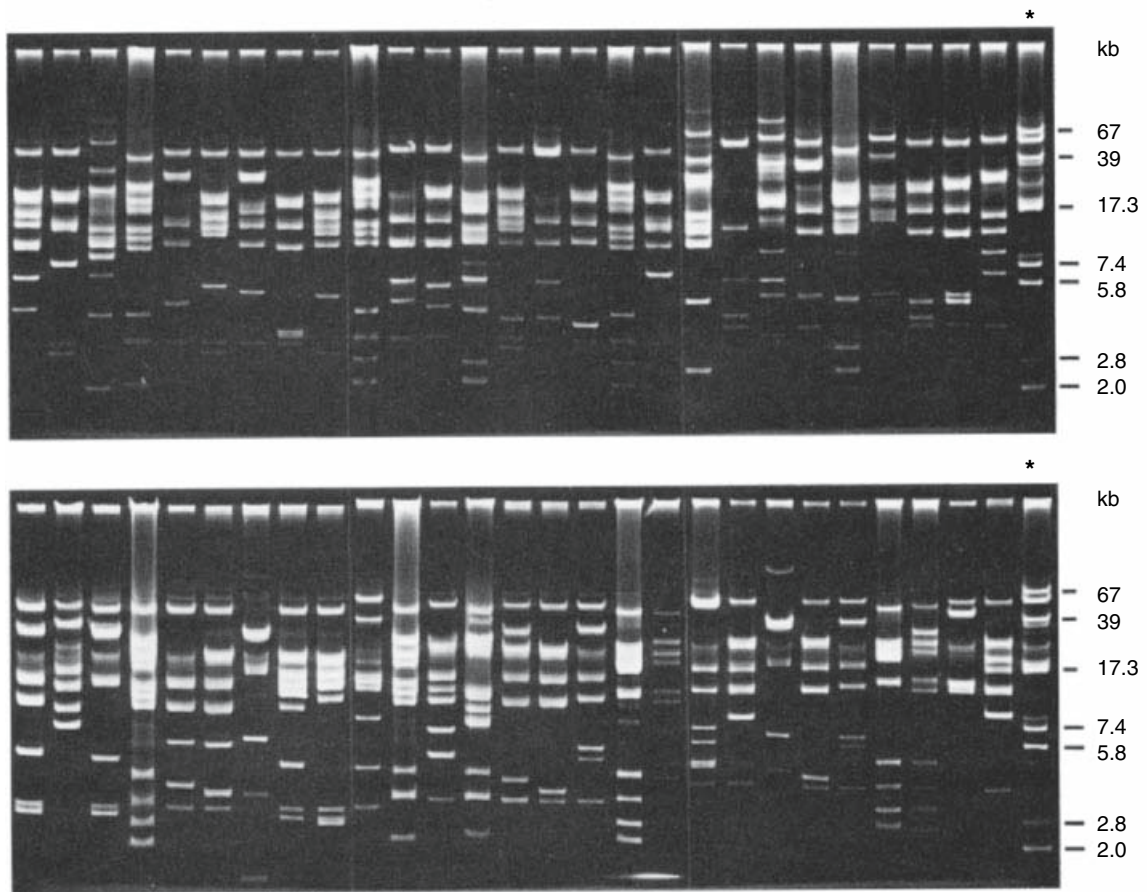
Fig. 6. Plasmid profiles of 54 *Lactococcus lactis* subsp. *lactis* strains isolated from an undefined mixed strain starter culture. Plasmid DNA from *L. lactis* subsp. *cremoris* AC1 (Neve et al., 1984) was used as size standard (indicated by asterisks on the right lanes). Electrophoresis was done on 0.8% agarose gels. (Adapted from Andresen et al., 1984.)
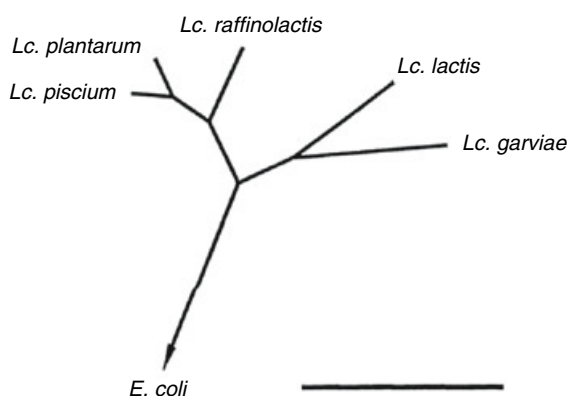
Fig. 7. Phylogenetic tree reflecting the close relationship within the genus *Lactococcus*. The tree was constructed by a maximum likelihood analysis of 16S rRNA sequences. The bar indicates 10% estimated sequence differences (from Teuber, 1995. Courtesy of Wolfgang Ludwig, TU Munich, Germany).

cheeses, fermented milks, cultured butter, and casein (see Table 1).

Functions of Lactococci for Milk Fermentation and Cheese Production

The biochemical and technological functions of lactococci necessary for milk fermentation and cheese production can be summarized as follows:

FORMATION OF LACTIC ACID FROM LACTOSE This early function in fermentation starts with the phosphorylation of lactose during transport via a specific, membrane-bound phosphotransferase system. The lactose-6-phosphate is split by a phospho-β-galactosidase into glucose and galactose-6-phosphate, which is further metabolized by the tagatose 1-phosphate pathway. L(+)-Lactate is the main endproduct of glycolysis. The resulting lowered pH values (4.0–5.6) as com-

pared to milk (6.6–6.7) prevent or retard growth of spoilage bacteria, especially clostridia, staphylococci, Enterobacteriaceae, and psychrotolerant Gram-negatives like *Pseudomonas*. If the isoelectric point of casein at pH 4.6–4.8 is approached, casein is precipitated. This effect is used to curdle milk in the production of cottage cheese, quarg, sour milk, yogurt and casein.

Starter bacteria for this purpose are *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* (besides lactobacilli and *Streptococcus thermophilus*).

FORMATION OF DIACETYL FROM CITRATE Diacetyl is the most characteristic aroma compound provided by *Lactococcus lactis* biovar "*diacetylactis*." It is derived from the citrate of milk (about 0.1%) present in solution and in the casein micelles as a casein-citrate-calcium-phosphate complex. The pathway goes through oxaloacetate, pyruvate, and α-acetolactate with a coupled release of carbon dioxide, which induces eye formation in cheese but also unwanted floating of the curd in the manufacture of cottage cheese or quarg if an unbalanced mixed starter is used (see Table 1).

LIMITED PROTEOLYSIS DURING CHEESE RIPENING Many strains of all lactococcal species possess a β-casein specific, cell-wall-associated protease, together with a complement of peptidases including an aminopeptidase, an X-prolyl-dipeptidyl aminopeptidase, and a dipeptidase, which are necessary for growth in milk and may also function during cheese ripening (Thomas and Pritchard, 1987; Kok and Venema, 1988a; Kiefer-Partsch et al., 1989).

Propagation and Preservation of Starter Cultures

Propagation and preservation procedures are described in Teuber et al. (1995), Cogan and Accolas (1996), Sandine (1996), and Teuber (2000). Starter cultures are needed and applied by the industry producing food and feed as indicated in Table 1. Dairy products are almost always made with commercial cultures. Liquid, dried, and frozen starter cultures are in use. Microorganisms in starter cultures must have a high survival rate and optimum activity for the desired technological performance, e.g., the fermentation of lactose to lactate, controlled proteolysis of casein, and production of aroma compounds like diacetyl. Because the genes for lactose and citrate fermentation as well as for certain proteases are located on plasmids, continuous culture has not been successful because fermentation-defective variants develop easily. In most instances, pasteurized or sterilized skim

milk is the basic nutrient medium for the large-scale production of starter cultures because it ensures that only lactococci fully adapted to the complex substrate, milk, will develop. For liquid starter cultures, the basic milk medium may be supplemented with yeast extract, glucose, lactose, and calcium carbonate. To obtain optimum activity and survival, it may be necessary to neutralize with sodium or ammonium hydroxide the lactic acid that is produced. Because many strains of lactic streptococci produce hydrogen peroxide during growth under microaerophilic conditions, it has been beneficial to add catalase to the growth medium, thus leading to cell densities of more than 10^{10} viable units per ml of culture.

For the preparation of concentrated starters, the media are clarified by proteolytic digestion of skim milk with papain or bacterial enzymes to avoid precipitation of casein in the separator used to collect the lactococcal biomass. The available self-cleaning clarifiers, e.g., bactofuges, concentrate the fermentation broth to cell concentrations of about 10^{12} CFU per ml. These concentrates can either be lyophilized or are preferentially transferred dropwise into liquid nitrogen. The formed pellets are packed in metal cans or cartons and are kept and shipped at –70°C (dry ice). These modern starter preparations can be added directly to cheese vats because the starter bacteria are in late logarithmic growth phase and will consequently immediately resume logarithmic growth. This modern technology shifts most of the microbiological work and responsibility from the cheese factory to the starter producer.

The classical liquid starter culture with about 10^9 CFU per ml and traditional lyophilized cultures containing about 10^{11} CFU per gram must be further propagated in the factory. However, lyophilized cultures can be transported easily and kept at ambient temperature for several months. Although concentrated lyophilized starters are also suited for direct vat inoculation, their use involves a short lag phase before growth is resumed. Direct vat inoculation may have the advantage of protecting against bacteriophages, which are common in the open dairy fermentation systems (see below). Figure 8 gives a flow sheet for the production of modern starter cultures for direct vat inoculation.

The Bacteriophage Problem in Dairy Fermentation

The Extent of the Problem

Because sour milk and cheese are normally not made from sterilized milk and because most

Fig. 8. Schematic outline for the industrial production of modern freeze-dried, deep-frozen, concentrated starter culture preparations suitable for direct vat inoculation (for more details and references, see Teuber, 1995, and Cogan and Accolas, 1995).

pure seed culture

inoculum culture
▼
preculture
▼
production culture
in sterile medium
with pH control
▼
harvest and
concentration
of biomass by
centrifugation

cryo-protection		cryo-protection
freeze drying	pelleting into liquid nitrogen	filling into cans
grinding	packaging in cartons	shock freezing in liquid nitrogen at about −190°C
blending of different strains	storage at −60°C	storage at −60°C
packaging		
storage at 4°C		
shipping	shipping in dry ice	shipping in dry ice
direct vat inoculation	direct vat inoculation	direct vat inoculation

cheese fermentations are open systems (open vats, control of fermentation by direct and open inspection with hands, tools, etc.), bacteriophages are a common threat. (For a review of this problem, see Teuber, 2000.) Even pasteurized milk may still contain virulent bacteriophages and residual lactic acid bacteria.

The phages of the mesophilic dairy lactococci have been investigated in detail. At least 11 genetically distinct phage types have been described (Jarvis et al., 1991). They may be lytic or temperate. Many starter culture strains of lactococci carry prophages that may be released during sour milk and cheese fermentations. The microbiology and genetics of these phages have been investigated thoroughly, leading to clear information on phage adsorption, injection, replication, and release. The host specificities have been found to vary substantially. Bacteriophage-resistant mechanisms have been characterized as being due to inhibition of adsorption, DNA-injection, restriction/modification, or abortive infection. Many of these traits are coded for on plasmids and are therefore amenable to genetic engineering and transfers from cell to cell. In

cheese factories, high bacteriophage levels in whey and curds are found under the following conditions, which may also lead to contamination with undesirable bacteria (Everson, 1991): 1) Use of one centrifuge for whey and milk separation; 2) use of whey cream for milk standardization, whey transfers by pumps, and casein fine savers in the area of raw milk silos, pasteurization equipment, and milk vacreators (gas removal devices); 3) starter tanks located in the cheese vat room and entrance of bacteriophage to the tanks by air replacement during cooling or emptying; 4) separate facilities for starter cans that have not been pressurized; the movement of air from whey handling areas to raw milk storage or cheese vat areas; 5) whey stored in lines connecting vats (must be water- or air-flushed between vats); 6) starter culture medium stored in lines (which should be kept at a minimum length) connecting vats or lines from the pasteurizer and starter room; 7) floor drains in the starter room (which should be eliminated) and drains in the cheese production room improperly flushed and sanitized during separate intervals of the day; 8) improper cleaning of cheese vats

between fills and lack of use of a sanitizer with at least 50–100 ppm available chlorine in the vat clean-in-place (CIP) system. Rinsing of vats between refilling using whey rather than pure water contributes to a bacteriophage build-up and should be avoided; 9) use of whey evaporator condensate or reverse osmosis permeate to wash tanks and cheese-making equipment; 10) use of long (excess of 1 hour) preripening times; 11) use of non-phosphate-buffered media for bulk starter propagation; and 12) use of single-strain starter culture, if not properly protected.

In addition to the use of phage-resistant strains developed by genetic engineering (Christian Hansen) or selected by challenge with bacteriophage cocktails (Müler and Teuber, 1988), there are many generally accepted technological measures that reduce or avoid the bacteriophage problem. These include starters containing phage-unrelated or phage-insensitive strains, production of phage-free bulk starter, aseptic propagation systems, phage-inhibitory media, reduction of phage contamination of processing plants, starter culture rotations, air conditioning and free air flow, avoidance of aerosol generation, cleaning/chlorination of vats between refills, use of clean-in-place (CIP) systems, segregation of starter room and cheese process equipment, removal of deposits on bulk starter vessels, good factory design (suitable location of whey storage tanks, whey handling systems, and wastewater treatment plants), regular use of an aerosol containing an agent such as chlorine to reduce contamination in the atmosphere, and other measures such as use of cultures for direct setting of the milk, inspection of jacket/agitators for pinholing, and early renneting.

Slow acidification due to bacteriophage attack of the starter culture may lead to good proliferation conditions for undesirable contaminant bacteria such as staphylococci, enterococci, listeria, enteric bacteria, and others. The use of strains resistant to specific phages may induce the selection of new, less virulent phages not encountered previously (Moineau et al., 1993). The proper handling of the bacteriophage issue still remains a major challenge for the maintenance of proper dairy fermentations.

The *Lactococcus*-Specific Bacteriophages

Bacteriophages are a common and constant threat to milk fermentation by lactococci, as most fermentations cannot be performed under sterile conditions because heat sterilization damages the casein micelles, preventing proper coagulation and syneresis of the cheese curd. Even pasteurized milk may still contain phages and some residual lactic acid bacteria. Hundreds of such phages have been isolated and described

during the last five decades (see Hill, 1993 and Teuber, 1995, for a review). However, only after progress in molecular biology could these phages be differentiated on the basis of, for example, DNA-DNA hybridization and protein profiles. As a consequence, the International Subcommission on the Taxonomy of Bacteriophages of *Lactococcus* was able to agree on the establishment of defined bacteriophage species and type phages, which are available from an international reference laboratory (Braun et al., 1989; Jarvis et al., 1991).

The 11 defined species (see Table 7) do not show detectable DNA similarity, which is quite astonishing on the basis of the quite similar morphology especially of species commonly named "small isometric head" phages. Most of these species have been isolated as virulent or lytic phages. Only BK5-T is a temperate phage, and P335 reveals strong DNA similarity with some temperate phages. Between 50 and 70% of the investigated bacterial host strains could be induced to release between 1 and 3 morphologically distinct prophage particles upon induction with mitomycin C or UV irradiation (Teuber and Lembke, 1983; see Fig. 9).

The complete nucleotide sequence of *L. lactis* subsp. *lactis* IL1403 revealed the presence of 6 prophages in this genome (Chopin et al., 2001). Isolation of prophage-cured strains, however, is extremely difficult because of immediate relysogenization of the cured derivatives in the induction assay. By electron microscopy, at least 19 morphologically distinguishable prophage particles have been detected. The taxonomic diversity of the lactococcal bacteriophages suggests, together with the observed diversity of lactococcal strains in mixed-strain starter cultures, a long and rapid evolution.

Biology of Host-Virus Interaction

Typically, milk to be fermented with *Lactococcus* is seeded with about 10^7 CFU ml^{-1}, which grow up to about 5×10^9 CFU ml^{-1} at the end of fermentation. Nine cell divisions will yield this increase. If only half of the world's cheese production is assumed to be manufactured with lactococci (i.e., 7.5 million tons or 7.5×10^9 kg), this corresponds to 7.5×10^{10} liters of milk inoculated with about 10^{21} CFU proliferating to 10^{23} viable bacteria every year. Because cheese whey from a mixed-strain fermentation typically contains 10^6–10^8 phage particles ml^{-1} (even in the absence of a noticeable phage attack), up to at least 10^{21} phage particles are released during roughly 10^{22} cell divisions. This again provides ample opportunities for evolution of bacteria and phages. Phage adsorption is usually not equally and densely packed over the whole bacterial cell sur-

Table 7. Phage species, type phages and some characteristics of lactococcal bacteriophages.*

Family	Morpho-logical type	Phage species	Type phage	Morphology			Appendages, unusual features	Phage genome		
				Head diameter	Tail diameter	Tail length		Size (kb)	Cohesive or noncohesive ends	G + C (%)
1. Siphoviridae	B1	936	P008	53	11	159	Collar, whiskers	29.7	C	37.5
2. Siphoviridae	B1	P335	P335	54	11	145	Collar, large base plate	36.4	NC	
3. Siphoviridae	B1	P107	P107	55	11	152	Long tail fiber	51.5		
4. Siphoviridae	B1	1483	1483	52	10	127		36	NC	
5. Siphoviridae	B1	P087	P087	65	16	200		54.5		
6. Siphoviridae	B1	1358	1358	53	10	109	Distinctive cross bars on tail	40	NC	
7. Siphoviridae	B1	BK-T	BK5-T	58	11	233		37.6	NC circular	
8. Siphoviridae	B1	949	949	79	14	527		52		38.8
9. Siphoviridae	B2	C2	c6A	60 × 41	10	87		21.9		36.7
10. Podoviridae	C2	P.34	P.34	65 × 44	10	24	Short tail collar with fibrils	18.1		
11. Podoviridae	C3	KSY1	KSY1	220–260	5	25–35	Elongated head, short tail triple collar with spikes			

*Modified from Jarvis et al. (1991).

virulent phage groups   temperate phage groups
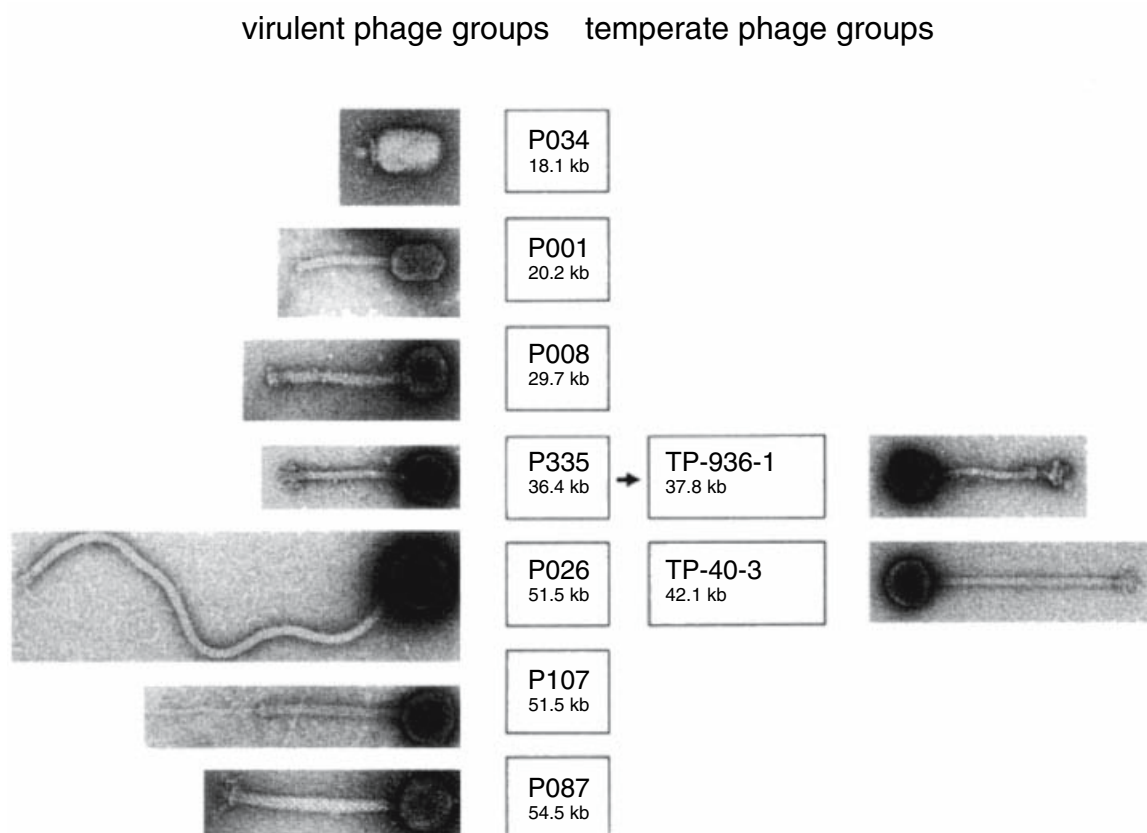
Fig. 9. Electron micrographs of virulent and temperate lactococcal-type phages with their corresponding genome sizes. The arrow indicates genetic relationship between virulent and temperate phages. (Adapted from Braun et al., 1989.)

face, but to a few specific spots at the surface, possibly in the vicinity of the plane of cell division (Budde-Niekiel and Teuber, 1987). For that reason, the biochemical identification and isolation of phage receptor molecules has not yet been successful, although membrane lipoteichoic acid, cell-surface galactose- and rhamnose-containing polysaccharides, and murein complexes have been suggested to participate in phage adsorption. Latency periods after infection are around 90 min and burst sizes between 10 and 100 per infected cell (Neve and Teuber, 1991).

Most isolated virulent phages are very specific for a limited number of strains; a few, especially the prolate-headed phage species c2, may even cross the subspecies line between "*lactis*" and "*cremoris*." In addition, bacteria infected and lysed by such phages produce a highly active bacteriophage-coded lysin, which is able to lyse lactococci from the outside, even if the strain is resistant to phage adsorption. For temperate phage particles released by mitomycin or UV induction from mixed-strain starter culture isolates, it is difficult to find sen-

sitive host strains in that and in other starter cultures. This may reflect in part the situation that prophage-sensitive bacterial strains are immediately and constantly eliminated in a starter culture either by lysis or by lysogenization. *Lactococcus lactis* subsp. *cremoris* Wg2, a strain well known from investigations of the plasmid-coded cell wall-protease system, is exceptionally susceptible to many prophage particles (possibly because of lack of a restriction/modification system).

Distribution of Bacteriophage Resistance

When single isolates (23 each) of three undefined mixed starter cultures (A, B and C) were tested against 375 phage strains from our collection, only 34 phages (i.e., fewer than 10%) showed lytic activities against one or more of the 69 bacterial strains. In all, 9 bacterial strains from culture A, 14 from culture B, and 2 from culture C were susceptible to one or more phages, the most susceptible being one strain from culture A, which was sensitive to 8 different phages (Budde-Niekiel et al., 1985). This observation

suggests that phage-host interactions are rather specific. This is also evident from the specificity of phages isolated during the application cycles of the three starter cultures in a cheese factory. The phages appearing during cycle A were mainly specific for bacterial strains from culture A, and likewise for the cycles B and C. The distribution of strains with varying degrees of phage resistance in four different, undefined mixed strain starter cultures is remarkable. A significant individual resistance level is evident. From a total of 7 undefined mixed-strain starters, 30 permanently and highly phage-resistant strains were obtained by challenging with a phage cocktail containing all phages ever isolated in the authors' laboratory (Müller and Teuber, 1988). This proves the high potential of lactococci to develop phage-resistant mechanisms, which actually allowed the successful use of such bacteria in spontaneous milk fermentations during the hundreds of years before the invention of starter cultures 100 years ago by Weigmann and others.

If the spatial distribution of phages in dairy plants is investigated, high numbers are found in whey and in the product (if it is not pasteurized). After enrichment, phages are detected in raw and pasteurized milk, on the surface of equipment, the clothes and skin of the cheese makers, and in the air, especially in the neighborhood of areas where whey-containing aerosols are produced (for example, close to whey separators). Cold disinfection of phage-contaminated surfaces and material is most effective with hypochlorite and peracetic acid (Lembke and Teuber, 1981).

Mechanisms of Bacteriophage Resistance

See Brüssow (2001) for a review of this topic. Restriction of bacteriophages can be demonstrated commonly in lactococci, e.g., with host-virus systems isolated from cheese factories. The observed efficiencies of restriction in plaque numbers are between 10^3 and 10^7. In addition, inhibition of phage adsorption has been reported quite regularly. However, because phage adsorption to lactococcal cells is difficult to determine and to distinguish from unspecific binding, owing to the "hot-spot" mechanism, adsorption studies can only be trusted if controlled by electron microscopic observation. The most interesting phenomenon is the quite common effect of "abortive infection." This has been demonstrated unequivocally by the proof of phage adsorption and DNA-injection with phages carrying a genomic DNA labeled with the fluorescent dye 4′,6-diamino-2-phenylindole dihydrochloride (DAPI; Geis et al., 1992). In some systems, phage replication is inhibited as late as in the transla-

tion of phage messenger RNA into phage protein.

Plasmids and Bacteriophage Resistance

Plasmids are common in lactococci as discussed above. In ecological terms, lactococci are able to exchange genes necessary for better survival in milk. Phage-resistant plasmids of lactococci have been characterized, and some of the genes coding for phage resistance have been cloned. In addition, phage-resistant plasmids have been transferred by conjugation to phage-sensitive starter culture strains. In the dairy industry in the United States, use of strains showing resistance to phage species c2 and 936 has led to the enrichment of P335 phages in American dairies. These newly isolated phages (known in Europe for some time) had high numbers of restriction sites in their genome, in contrast to the traditional c2 and 936 phage species. A low number of or no restriction sites is suggested to be the result of selection during evolution and the means by which these strains are able to escape the restriction/modification systems of lactococci (Moineau et al., 1993).

Molecular Biology of Lactococcal Bacteriophages

The study of the molecular biology of the bacteriophages of lactococci is in a rapidly developing stage. All phages hitherto isolated contain double stranded DNA in a closed circular, linear, or permutated form. We know the DNA nucleotide sequences of some genomes of temperate and lytic phages, allowing further insight into the modular construction, the replication and integration of these phages (e.g., phages c2, sk1, and bIL170). Work has been done regarding the integration of temperate phages into the lactococcal chromosome. Also, the characterization of restriction/modification systems involved in phage resistance is in progress. An interesting approach is the use of antisense mRNA to induce bacteriophage resistance in lactococci (for a detailed discussion and interpretation, see Brüssow, 2001).

Stability of Bacteriophage Resistance in Lactococci

The fact that plasmids code many resistance phenomena suggests that plasmid loss could lead to loss of phage resistance, which is indeed the case. Stadhouders and Leenders (1984) showed unequivocally that phage resistance of undefined mixed-strain cultures of everyday cheese making could be lost. As soon as the cultures were propagated in the laboratory in sterile skim milk in

the absence of phages, the mixed-strain cultures were converted into a culture with just a few dominating strains. The previously high bacteriophage resistance, known from cheese fermentations, was completely lost within a short time. This observation emphasizes the need for a constant phage challenge to such cultures under manufacturing conditions to keep them in an optimal state.

Genome Analysis

The detection of extrachromosomal elements (plasmids) in lactococci (Cords and McKay, 1974) and the very recent presentation of the complete genome sequence of *Lactococcus lactis* IL1403 (Bolotin et al., 1999; Bolotin et al., 2001) are two investigative milestones in the genetics of these bacteria in the last 20 years.

Lactococcus lactis is an A+T-rich Gram-positive bacterium; its genome consists of 2345 ± 5 kb with an average G+C-content of 35.4%. The sequencing data allowed the prediction of some very interesting features of the lactococcal genome. The presence of 42 copies of the 5 different insertion (IS)-elements—2 of the 5 are unknown so far—indicate the importance of these elements for genetic exchange. Besides this large number of IS-elements, the genome of IL1403 carries six potential or rudimentary prophages (Chopin et al., 2001). The genome sequencing data confirmed the occurrence of six rRNA operons.

By use of the specific computer program tRNAscan-SE (Lowe and Eddy, 1997), 62 tRNA genes were detected, the majority organized in 4 large operons. Comparisons of the genome sequence with entries of databanks allowed the identification of 1495 protein encoding genes which were mainly colinear to the replication direction. The complete lactococcal genome was estimated to be about 2300 genes, approximately one gene per kb.

Amino Acid and Protein Metabolism

Dairy lactococci depend on the presence of some essential amino acids for growth (Anderson and Elliker, 1953; Jensen and Hammer, 1993), whereas some *Lactococcus lactis* subsp. *lactis* strains isolated from non-dairy environments are prototrophic for amino acids. The analysis of the genome sequence showed that *L. lactis* strain IL1403 has the genetic potential (95 putative genes) to synthesize all 20 standard amino acids. Mutational inactivation of some amino acid biosynthetic pathways, a possible consequence of

the adaption to milk, can explain the auxotrophic phenotype of dairy lactococci. Several operons involved in the biosynthesis of amino acids (e.g., aromatic amino acids, histidine, threonine and the branched amino acids; Bardowski et al., 1992; Delorme et al., 1993; Godon et al., 1993; Griffin and Gasson, 1995; Madsen et al., 1996) have been characterized in detail in lactococci from dairy and non-dairy origins. The genes for the biosynthesis of a given amino acid are clustered in operons, which are tightly regulated by termination/antitermination or transcriptional attenuation mechanisms. The regulatory genetic elements, transcription promoters and terminators, and ribosomal binding sites are generally similar to those of other bacteria.

Owing to the amino acid auxotrophy and the lack of sufficient amounts of free amino acids in milk, lactococci need a proteolytic system for growth in milk. This system consists of a cell-wall bound protease (PrtP), three peptide transporters (oligopeptide permease [Opp], DtpT, and DtpP) and a variety of peptidases (Pep). The genes for the majority, if not all, of the enzymes necessary for casein degradation and transport of the degradation products were cloned, sequenced and analyzed in detail. From these data, the following picture emerges. The caseins of the milk were partially degraded by the cell-wall bound protease into a large number of oligopeptides, some of which (≥10 amino acid residues) were taken up by the oligo- and the di/tripeptide transport systems and subsequently hydrolyzed to amino acids by a plethora of peptidases. Mutants missing transporter and/or peptidase genes have been constructed by deletion or disruption of the corresponding genes. Those missing the Opp system but still having di/tripeptide transport activity are unable to grow in milk. Mutants with an increasing number of peptidase mutations showed decreasing growth rates in milk—a five-fold peptidase mutant grew at one-tenth the wild-type rate. The genes for the peptide transport systems and the peptidase were located on the chromosome, and those for the proteases exclusively on plasmids (Kok and de Vos, 1994; Kunji et al., 1996; Mierau et al., 1997; Christensen et al., 1999; Siezen, 1999).

Plasmids

Lactococci possess an unusually large complement of plasmids (Fig. 6), ranging in molecular size from about 2 to more than 100 kb. The number of plasmids from different strains isolated from dairy cultures varies from one to 12 (Cords et al., 1974; Pechmann and Teuber, 1980; Gasson, 1983; Andresen et al., 1984). Extrachromosomal DNA represents up to 10% and even more of the

coding capacity of lactococci. Plasmids can be transferred among lactococci in their natural habitats and even into other bacteria by conjugation and mobilization (Gasson and Davies, 1980; Walsh and McKay, 1981; Neve et al., 1984; Neve et al., 1987; Gasson, 1990). Transfer of plasmid DNA by transduction can easily be performed in experimental systems (Allen et al., 1963; McKay et al., 1973; Fitzgerald and Gasson, 1988). Whether this type of gene transfer also occurs naturally is an open question.

Plasmid-curing by treatment with acridine dyes, ethidium bromide, nalidixic acid, growth at elevated temperature, or protoplast regeneration provided the first evidence for plasmid linkage of a variety of technologically important fermentative functions (Kuhl et al., 1979; Pechmann and Teuber, 1980; Gasson, 1983; Geis et al., 1983). As already mentioned, the gene for the cell-wall bound protease (PrtP), essential for growth in milk, is exclusively encoded on plasmid DNA. The gene for PrtP has been cloned and sequenced for a number of *L. lactis* strains (de Vos et al., 1993; Kok et al., 1988b; Kiwaki et al., 1989). The primary sequences of the lactococcal enzymes are more than 98% identical, but the corresponding proteases can be divided into two main specificity classes, PI and PIII (Visser et al., 1986). Enzymes of the PI type degrade β-caseins and to a lesser extent k-casein, whereas those of the PIII type are able to hydrolyze in addition α_{s1}-casein (Prichard and Coolbear, 1993). The lactococcal proteinases show close homology to subtilisins, which are serine-proteases with similar catalytic domains. Post-translational processing of the primary gene product (approximately 200 kDa) includes removal of an N-terminal signal (a 33 amino-acid-residue sequence) and a prosequence (of 154 amino acid residues), resulting in mature PrtP-protein fixed to the cell wall by its C-terminus. In proximity to the PrtP-gene, an oppositely directed gene (*prt*M) was identified, encoding for a lipoprotein essential for the protease processing (Haandrikman et al., 1989; Haandrikman et al., 1991).

Lactose Metabolism

Lactococci are homofermentative lactic acid bacteria. During growth in milk, lactose is converted to lactic acid. Lactose is taken up by a phosphoenol pyruvate (PEP)-dependent phosphotransferase system (PEP-PTS^{Lac}-lac A,B). During transport, lactose is phosphorylated to lactose-6-phosphate, which is subsequently hydrolyzed by a phospho-β-galactosidase (lacG) into glucose and galactose-6-phosphate (Gal-6-P). Glucose is phosphorylated by glucokinase and metabolized to lactate by the glycolytic pathway. Gal-6-P is converted to the glycolytic intermediates (glyceraldehyde 3-phosphate and dihydroxyacetonephosphate) by enzymes of the tagatose pathway, including galactose 6-P isomerase (lacAB), tagatose 6-phosphate kinase (lacC) and tagatose 1,6-diphosphate aldolase (lacD). So far, the lac-phenotype was always found to be correlated to the presence of a specific plasmid. In *L. lactis* subsp. *cremoris* NCDO712, the structural genes for the lac-specific PTS-system, the phospho-β-galactosidase, and the enzymes of the tagatose pathway are organized in a 7.8-kb lac-operon:lacABCDFEGX located on a 56.6-kb conjugative plasmid. The oppositely orientated gene for a specific repressor (lacR) is located immediately upstream of lacA. This gene is expressed in the presence of glucose and repressed during growth in milk. Transcription of the lac operon into two polycistronic transcripts (lacA–E and lacA–X) is regulated by the repressor lacR and tagatose 6-phosphate as inducer (van Rooijen and de Vos, 1990; van Rooijen et al., 1991; van Rooijen et al., 1992).

Bacteriocins

Bacteriocins, proteinaceous compounds that kill closely related bacteria, are produced by a variety of lactococcal strains. In a survey of 280 strains isolated from dairy environments, 5% were found to produce such substances. On the basis of biochemical and physical properties, host range, and crossreactivity, eight bacteriocin types were predicted (Geis et al., 1983; Klaenhammer, 1993). In recent years, a number of these bacteriocins has been characterized by genetic and biochemical methods. These belong mainly to two defined classes: lantibiotics-class I, and small, heat stable non-lantibiotics-class II (Nes et al., 1996).

Nisin, a class I bacteriocin, is a small peptide (34 amino acid residues) produced by several *L. lactis* subsp. *lactis* strains (Hirsch, 1953; de Vos et al., 1993). It strongly inhibits the growth of a broad range of Gram-positive bacteria. Two natural variants, nisin A and Z were found (Gross and Morell, 1971; Mulders et al., 1991). Nisin is ribosomally synthesized as a 57-amino acid precursor peptide, which is subjected to various modifications. The mature peptide (34 amino acid residues) shows some unusual features such as the dehydrated amino acids dehydroalanine and -butyrine, as well as lanthionine and β-methyllanthionine residues, which form five intracellular thioester bridges (Gross and Morell, 1971; Jung, 1991). Nisin is able to form pores in the membrane of Gram-positive bacteria mediating the efflux of ions, amino acids and ATP from cells (Sahl et al., 1987). The structural genes for nisin

A and Z were cloned and sequenced (Buchman et al., 1988; Kaletta and Entian, 1989; Dodd et al., 1990; Mulders et al., 1991; Rauch and de Vos, 1992). The structural genes are part of a large gene cluster of eleven genes which are involved in all aspects of the nisin biosynthesis. The following, autoregulated pathway for nisin has been suggested. Via a two-component signalling system, nisin activates the nisA promoter. This results in the production of prenisin, which is modified by a membrane-bound enzyme system. Subsequently the precursor nisin is translocated by an ABC transporter and activated by proteolytic cleavage by an extracellular proteinase.

Nisin production is always linked to the ability to ferment sucrose (Hirsch and Grinsted, 1951; Rauch and de Vos, 1990). This linkage was confirmed by curing experiments and conjugal transfer (Gasson, 1984; Gonzales and Kunka, 1985; Steele and McKay, 1986). It could be shown that the nisin-sucrose element is part of a conjugative transposon that integrates into several different sites of the lactococcal chromosome by a mechanism of transposition similar to that of the Tn916 family (Horn et al., 1991; Rauch and de Vos, 1992; Thompson et al., 1991).

A number of class II bacteriocins have been detected and analyzed biochemically and by genetic methods (van Belkum et al., 1989; van Belkum et al., 1991; van Belkum et al., 1992; Holo et al., 1991; Nissen-Meyer et al., 1992). The class II bacteriocins can be divided in some subgroups but share a number of common features. They have a high content of small amino acids such as glycine, are strongly cationic (pI 8–11), and possess a hydrophobic domain. Some of the bacteriocins consist of two peptides whereby the bacteriocidal effect depends on the complementary action of both components. *Lactococcus lactis* strains can produce up to three different bacteriocins. Bacteriocin producers have an immunity system which protects the cells against its own bacteriocin. Each bacteriocin has its own immunity factor (fairly small proteins of 50–150 amino acids). The exact function of the immunity factors is still not understood. Class II bacteriocins interact strongly with the negatively charged phospholipids of the cell membrane and form pores which dissipate the proton motive force. The genetic determinants for bacteriocins were found on plasmids and in general consist of four genes: 1) the structural gene encoding a prebacteriocin, 2) an immunity gene proximal to the structural gene on the same transcriptional unit, 3) a gene encoding an ABC-transporter which processes the prebacteriocin and excretes the mature bacteriocin, and 4) a gene encoding an accessory protein of unknown function. The genes can be in one or two operons (Nes et al., 1996; Nes and Holo, 2000).

Exopolysaccharides

The production of significant amounts of capsular- or exopolysaccharides (EPS) is quite common among Gram-positive bacteria. In recent years, a growing number of *Lactococcus lactis* strains were found to produce EPS (Neve et al., 1988; Vedamuthu and Neville, 1986; von Wright and Tynkkynen, 1987). In one study, the chemical composition of EPS in 16 of these strains, as well as the localization, sequences, and organization of the *eps* genes involved in EPS biosynthesis were analyzed. The strains could be divided into three major groups. Group I (six strains) produced EPS containing galactose, glucose and rhamnose. Group II (five strains) produced EPS with only galactose, whereas group III (three strains) made EPS with galactose and glucose. Two strains showed unique EPS sugar composition (van Kranenburg et al., 1999). The genes for EPS synthesis were always found on plasmids larger than 20 kb and were clustered in large operons of up to 14 coordinately expressed genes; one of these plasmids, the 42,180-bp pNZ4000, has been completely sequenced (van Kranenburg et al., 2000).

Genetics

In the last 15 years, sophisticated genetic tools—efficient transformation and special vector systems—have been developed which allow analysis of gene regulation, cloning and expression of homologous and heterologous genes. Transformation of lactococci is routinely performed by electroporation (Harlander, 1987; Powell et al., 1988; Holo and Nes, 1989). Several protocols exist which allow efficient transformation even of strains insensitive to the formerly used polyethylene glycol (PEG)-induced protoplast transformation (Geis, 1982; Kondo and McKay, 1984; Simonet et al., 1986). A large number of vector systems now allow cloning and expression of genes from different sources, random and targeted inactivation of genes, selection for promoter, terminator, and signal sequences, and anchoring of proteins to the surface of lactococci (Kok et al., 1984; van der Vossen et al., 1985; de Vos, 1986; Simon and Chopin, 1988; van der Guchte et al., 1989; Maguin et al., 1996; O'Sullivan et al., 1996; Leenhouts et al., 1998; Poquet et al., 1998; Sorensen et al., 2000). Only the most sophisticated expression vector system that allows very efficient control of gene expression in *Lactococcus* and other lactic acid bacteria will be described (de Ruyter et al., 1996).

This system makes use of the autoregulatory properties of the nisin gene cluster. Nisin, at con-

centrations far below the minimal inhibitory concentration, acts as an external inducer via a two-component signal transduction system consisting of a histidine protein kinase (NisK) and a response repressor (NisR). The two genes (*nisK* and *nisR*) under the control of a constitutive promoter are delivered by recombinant plasmids or are integrated into the chromosome of the expression strain. The gene to be expressed is fused to the *nisA* promoter, which is part of the expression vector. Vectors for transcriptional and translational fusions have been constructed. Homologous as well as heterologous proteins representing up to 47% of the total cell protein can be produced with increasing amounts of the inducer.

In recent years, several systems to anchor and to display heterologous polypeptides at the surface of lactococci have been developed (Piard et al., 1997; Leenhouts et al., 1999). This capability allowed the surface composition of cells to be changed, a strategy that may help in the understanding of the mechanisms of protein targeting. Changes in the surface composition may also influence interactions between the bacteria and its environment and allow potentially important biotechnological applications such as immobilization of enzymes at the bacterial surface, fixing of cells to special carrier surfaces, and display of

entire peptide libraries. *Lactococcus lactis* presenting the appropriate antigens may in the future be used as live bacterial vaccine delivery systems (Norton et al., 1995; Robinson et al., 1997; Steidler et al., 1998).

By fusion of genes for heterologous polypeptides to genes of proteins known to be fixed to and presented at the cell surface, a variety of peptides and proteins could be anchored and at least partially presented at the cell surface of lactococci. These include enzymes (β-lactamase, Buist et al., 1997; α-amylase, Buist et al., 1997, and nucleases, Poquet et al., 1998), and epitopes of the human cytomegalovirus (Franke, 1998), human immunodeficiency virus (Leenhouts et al., 1999), the *Plasmodium falciparum* merocoite stage surface antigen (Leenhouts et al., 1999), and the *Clostridium tetani* toxin C-fragment (Norton et al., 1995; Norton et al., 1996).

Antibiotic Resistance

The molecular analysis of an antibiotic resistant strain of *L. lactis* subsp. *lactis* isolated from a raw milk soft cheese revealed a 29,815-bp multiresistance plasmid pK214 (Perreten et al., 1997). It is an assembly of resistance genes such as *tetS*, *str* (streptomycin adenylase), *cat* (chloramphenicol
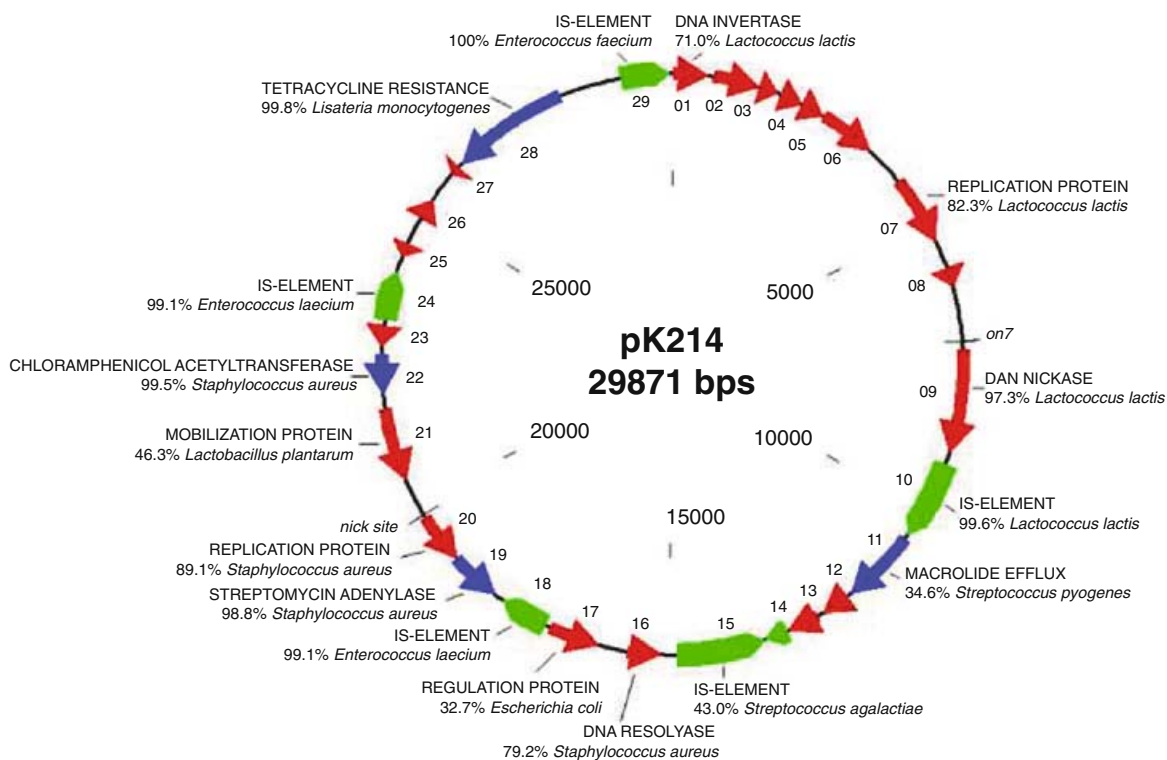
Fig. 10. Genomic structure of the multiresistance plasmid pK214 isolated from *Lactococcus lactis* subsp. *lactis* isolated from a raw milk soft cheese (Perreten et al. 1997; detailed discussion in Teuber et al. 1999).

acetyltransferase), and a new multiple drug transporter (*mdtA*), all of which have previously been detected in *Listeria monocytogenes* and *Staphylococcus aureus* (Teuber et al., 1999). The *mdtA* gene codes for a new efflux protein (Perreten et al., 2001). This observation proves that lactococci are able to pick up multiple antibiotic resistance elements in an antibiotic challenged environment. Figure 10 shows the structure of pK214.

Pathogenicity

Clearly, *L. garviae* must now be regarded as a fish pathogen and responsible for mastitis in cows and buffalos. It has been designated an emerging pathogen of increased clinical significance in both veterinary and human medicine (Facklam and Elliot, 1995; Vela et al., 2000). *Lactococcus garviae*, the etiologic agent of hemorrhagic septicemia in farmed trout, is characterized by bilateral exophthalmos, darkening of the skin, blood congestion in the vessels of the intestine, liver, kidney, spleen, and brain, and a characteristic hemorrhagic enteritis (Doménech et al., 1993). The disease has been termed "lactococcosis" to distinguish it from streptococcosis and occurs worldwide, affecting fish species as different as eels, yellowtails, farmed trouts, and prawns. *Enterococcus seriolocida* is a junior synonym of *L. garviae* (Eldar et al., 1999). Pathogenicity and virulence factors have not yet been identified.

In contrast, *L. piscium* seems to be a meat spoilage bacterium encountered in vacuum-packed, chilled meat (Sakala et al., 2002). The isolation of the type strain from a diseased rainbow trout (Williams et al., 1990) cannot be taken as evidence for primary pathogenicity because no other cases have been reported since.

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CHAPTER 1.2.8

The Genera *Pediococcus* and *Tetragenococcus*

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Introduction

The genera *Pediococcus* and *Tetragenococcus* are typical lactic acid bacteria (LAB) in being Gram positive, catalase negative, and oxidase negative. They grow under facultatively aerobic to microaerophilic conditions. They are homofermentative and produce lactic acid but no CO_2 from glucose and are not able to reduce nitrate. The uniformly spherical cells are never ovoid or elongated (Günther and White, 1961b), and they differ from all other LAB by forming tetrads via alternate division in two perpendicular directions (Fig. 1). Thus, they never form chains typical of the other genera of coccus-shaped organisms *Leuconostoc*, *Lactococcus* and *Streptococcus*, which form chains as a result of division in one plane. The name "*Pediococcus*" came from the suggestion by Balcke (1884), which was based on the observation that the cells divide in one plane (the Greek noun *pedium* means a plane surface). The genus *Pediococcus* (Claussen 1903) is incorrectly cited as *Pediococcus* (Balcke 1884) in the "Approved Lists of Bacterial Names" and also in the amended edition of the "Approved Lists of Bacterial Names" (Lapage et al., 1992; Euzéby, 1998). The type species is *Pediococcus damnosus*.

The genus *Pediococcus* is only distantly related to the genus *Tetragenococcus*, while geno- and phenotypically, it most closely resembles *Lactobacillus casei/paracasei* (Fig. 2). The genus *Aerococcus*, previously grouped with the pediococci because of morphological resemblance, is catalase positive and, on the basis of comparative 16S rRNA sequence analysis, phylogenetically closer to *Tetragenococcus*, together with the genera *Enterococcus*, *Carnobacterium*, *Vagococcus* and *Alloiococcus*. In the first edition of *The Prokaryotes*, the two genera *Pediococcus* and *Aerococcus* were treated in two different chapters: "The Family Streptococcaceae: Nonmedical Aspects" (Teuber and Geis, 1981) and "The Family Streptococcaceae: Medical Aspects" (Facklam and Wilkinson, 1981), respectively, and in only one chapter in the second edition (Weiss, 1992). Aerococci are also isolated from sources quite different from those of pediococci, such as from the air in hospital environments (Kerbaugh and Evans, 1968), from vegetation, outdoor air, and dust (Deibel and Niven, 1960), from human clinical specimens (Bosley et al., 1990), and as marine organisms causing fatal disease of lobsters (Hitchner and Snieszko, 1947; Wiik et al., 1986). The medical importance of aerococci as causative agents of different diseases such as osteomyelitis, endocarditis, and septic arthritis is documented by many case reports (Janosek et al., 1980; Facklam and Wilkinson, 1981; Taylor and Trueblood, 1985) but not fully understood (Weiss, 1992). Of the four presently recognized *Aerococcus* species (*Aerococcus sanguinicola*, *Aerococcus urinae*, *Aerococcus urinaehominis* and *Aerococcus viridans*), A. urinae and A. viridans are classified as risk group 2 organisms (Evans and Schultes, 1969; Nakel et al., 1971; Evans, 1986; Garvie, 1986; Christensen et al., 1991; Aguirre and Collins, 1992; Lawson et al., 2001; Facklam et al., 2003).

The genus *Pediococcus* comprises six validly described species, considered to be typical pediococci, viz.: *Pediococcus acidilactici*, *P. pentosaceus* with the subspecies *P. pentosaceus* subsp. *pentosaceus* and *P. pentosaceus* subsp. *intermedius*, *P. damnosus*, *P. parvulus*, *P. inopinatus* and *P. claussenii*. The atypical species *P. dextrinicus* produces L(+) lactic acid from glucose via a fructose-1,6-diphosphate FDP inducible L-lactate dehydrogenase (L-LDH; Back, 1978a) and will be suggested as representative of a new genus (W. Ludwig et al., manuscript in preparation). The reclassification of the species "*P. urinae-equi*," formerly considered to belong to the pediococci, as *Aerococcus viridans* has been suggested (Bergan et al., 1984; Stackebrandt and Teuber, 1988; Collins et al., 1990); this is supported by the tree topology of the small subunit (SSU) ribosomal RNA in Fig. 2, indicating a close relationship also with *Aerococcus urinae*, and the distance to the Lactobacillales and the true pediococci. The halophilic species "*Pediococcus halophilus*" has been assigned to the new genus *Tetragenococcus* (Collins et al., 1990). The key characteristics for differentiating the "true"
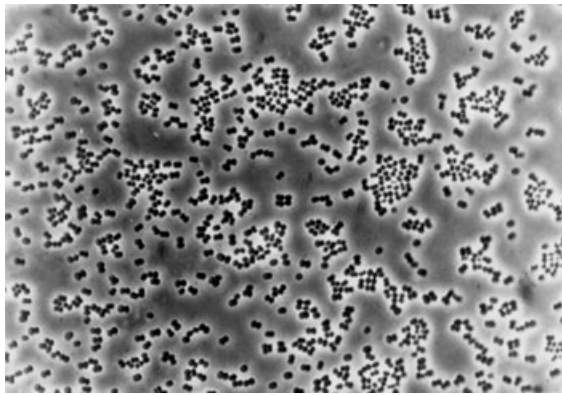
Fig. 1. Phase contrast photograph of *Pediococcus damnosus*; diameter of the individual cells, 0.7–0.8 μm.

pediococci from *Tetragenococcus*, *Aerococus* and *P. dextrinicus* are summarized in Table 1. With the exception of *P. claussenii* (the type strain of which produces L(+) lactic acid), all pediococci produce DL lactic acid as the major end product of glucose fermentation, as compared to the L(+) lactic acid isomer produced by *Tetragenococcus* and also by *P. dextrinicus*. Pediococci, and also *P. dextrinicus*, are typically aciduric (grow at pH 5.0), which distinguishes them from *Aerococcus* and *Tetragenococcus*. Two species are presently assigned to the genus *Tetragenococcus*, viz., *Tetragenococcus halophilus* (Collins et al., 1990; validated: Anon., 1993), and *Tetragenococcus muriaticus* (Satomi et al., 1997). Both of these species are typically associ-
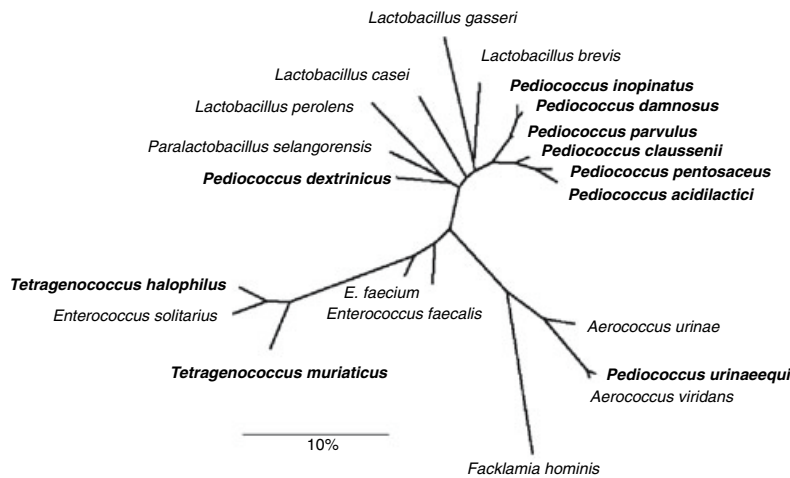
Fig. 2. Small subunit ribosomal rRNA-based tree reflecting the relationships of *Pediococcus*, *Tetragenococcus* as well as selected Lactobacillales species. The tree topology was reconstructed by applying the ARB-parsimony tool (Ludwig et al., 2004) to an (http://www.arb-home.de{ARB 16S rRNA database}) comprising 28,980 almost complete sequences. Alignment positions were included which are invariant among at least 50% of all representatives of Lactobacillales. Local tree topologies of the clusters comprising pediococci, tetragenococci and aerococci were optimized applying 50% conservation filters (Ludwig et al., 2004) to the true *Pediococcus*, Enterococcaceae, and Aerococcaceae, respectively. The topology is supported by distance matrix and maximum parsimony analyses of subsets of the sequence data using the respective ARB tools. Only type strains are shown. The length bar indicates 10% estimated sequence divergence.

Table 1. Key characteristics for differentiating the genera *Pediococcus* and *Tetragenococcus* from *Aerococcus* and the "atypical" species "*P. dextrinicus*."

Characteristic	*Pediococcus*	*Tetragenococcus*	*Aerococcus*	"*P. dextrinicus*"
Growth at pH 5	+	−	−	+
pH 9	−	+	+	−
Growth tolerance to 18% NaCl	−	+	−	−
Facultative aerobic	+	+	−	+
Gas from gluconate	−	−	−	+
Configuration of lactate from glucose	DL/L(+)	L(+)	L(+)	L(+)
Catalase	−	−	+/−	−
Hippurate hydrolysis	−	−	+	−
Starch fermentation	−	−	−	+
Peptidoglycan type	Lys-D-Asp	Lys-D-Asp	Lys-direct	Lys-D-Asp

Symbols: +, present; −, absent; and +/−, variable.
From Weiss (1992), Simpson and Taguchi (1995), and Dobson et al. (2002).

ated with fermenting brine mashes (see under Habitats).

Phylogeny

Comparative sequence analysis of 16S rRNA (Woese, 1987) is still the main basis for our present knowledge of bacterial phylogeny. However, other macromolecules, such as 23S rRNA (Ludwig and Schleifer, 1994), elongation factor Tu, and the β-subunit of ATPase (Ludwig et al., 1993), have been used to extend knowledge of the phylogeny of Gram-positive bacteria (particularly the "*Clostridium* branch" of Gram-positive bacteria) as a line of descent (phylum) with guanine plus cytosine (G+C) DNA content of less than 50 mol%, to which also the pediococci, *Tetragenococcus*, and other typical LAB belong.

In recent years, especially 16S and 23S rRNA or rDNA sequencing, 16S rRNA cataloguing, and DNA:DNA hybridization studies have contributed extensively to our understanding of the relatedness between the genera of LAB (Holzapfel et al., 2001). On the basis of 16S phylogenetic trees, the genera *Enterococcus*, *Carnobacterium*, *Vagococcus*, *Aerococcus*, *Tetragenococcus* and *Alloiococcus* appear to be more closely related to each other than to other genera of LAB (Holzapfel et al., 2001). The genera *Lactococcus* and *Streptococcus* are also relatively closely related, as are the genera *Leuconostoc*, *Oenococcus* and *Weissella*, which jointly constitute the so-called "*Leuconostoc* group." Together with *Pediococcus*, these genera share physiological relatedness and were in fact previously considered to be phylogenetically intermixed (Stackebrandt and Teuber, 1988). The 16S rRNA sequence data also show that the genera *Pediococcus* and *Lactobacillus* can be grouped into a single cluster. Studies of 16S rRNA cataloguing and sequencing have shown that *Pediococcus pentosaceus* and its nearest relative *P. acidilactici* should be placed in the phylogenetic cluster formed by the lactobacilli and the leuconostocs (Weiss, 1992).

On the basis of 16S rRNA sequencing, *Pediococcus dextrinicus* is distantly related to *L. casei* (Collins et al., 1991) and is moderately related to *L. perolens* and *Paralactobacillus selangorensis* but does not group with the other pediococci (Fig. 2). This is also supported by the fact that all aciduric, facultatively anaerobic species form a phylogenetically tight group, with only *P. dextrinicus* being somewhat peripheral (Weiss, 1992). The close phylogenetic relationship of these six *Pediococcus* species (and their distance to *Pediococcus dextrinicus* and the genus *Tetragenococcus*) is indicated by the topology of the

SSU ribosomal rRNA-based tree shown in Fig. 2. By its 16S rDNA sequence, *P. dextrinicus* appears to be only distantly related to the "true" pediococci (Dobson et al., 2002) and, as indicated before, may also be phenotypically distinguished from other *Pediococcus* spp. by, e.g., the production of L(+) lactate as end-product from glucose metabolism, and also by the production of CO_2 from gluconate. Although this species still belongs to the genus *Pediococcus*, genetic and phenotypic data support its allocation to a new genus. Suggestions have in fact been made earlier to group *P. dextrinicus* with *Lactobacillus* (Collins et al., 1990; Stiles and Holzapfel, 1997), but no formal request for this change has been made to date (Dobson et al., 2002).

The species formerly known as *Pediococcus halophilus* was re-classified by Collins et al. (1990) as the first species belonging to the novel genus *Tetragenococcus*. Moreover, *Pediococcus urinae-equi* was shown to be very closely related to *Aerococcus viridans* (more than 99.9% sequence similarity of the 16S rRNA), suggesting that *P. urinae-equi* is a member of the genus *Aerococcus* (Collins et al., 1990). Despite this close relatedness in the 16S rRNA sequence, no official opinion that *P. urinae-equi* should be transferred to the genus *Aerococcus* has yet been rendered (Dobson et al., 2002).

Although *P. acidilactici* and *P. pentosaceus* are difficult to separate using physiological tests (which may suggest a high degree of relatedness), they actually share low DNA:DNA relatedness (5–35% similarity; Back and Stackebrandt, 1978c; Dellaglio and Torriani, 1986; Simpson and Taguchi, 1995; Table 2). Nevertheless, these two species show a closer relationship to each other than to other *Pediococcus* spp. when considering their phylogenetic relationship based on 16S rDNA gene sequence analysis (Fig. 2). *Pediococcus damnosus*, on the other hand, shows a high degree of genomic relatedness to *P. inopinatus* (41–54%) and *P. parvulus* (34–36%) in DNA:DNA hybridization experiments (Table 2). This is also reflected in the phylogenetic relatedness based on 16S rDNA sequences (Simpson and Taguchi, 1995; Dobson et al., 2002; Fig. 2). Dobson et al. (2002) used three different comparative phylogenetic sequence analyses, comprising the 16S rRNA gene, 16S-23S internally transcribed spacer regions, and the heat shock protein (HSP)60 gene for investigating the phylogenetic relationship between pediococci. In their investigations, they could confirm by all three methods that *P. pentosaceus* and *P. acidilactici* were closely related. Similarly, *P. damnosus* showed a high relatedness to *P. parvulus* and *P. inopinatus*. The three methods were excellent for delineating the species of the genus, and congruence of data

Table 2. Percent DNA-DNA similarity among pediococci.

	P. acidilactici	P. damnosus	P. dextrinicus	P. inopinatus	P. halophilus	P. parvulus	P. pentosaceus	P. pentosaceus subsp. intermedius	P. urinae-equi
P. acidilactici	100	0–7	0–5	0–7	0–2	0–7	5–35	17–19	0
P. damnosus	0–7	100	4–5	41–54	0–2	34–36	0–18	0–7	0
P. dextrinicus	0–5	4–5	100	7	6	8	6	5	0
P. inopinatus	0–7	41–54	7	100	3–5	30–40	7–8	6–7	0
P. halophilus	0–2	0–2	6	3–5	100	4	4	3	0
P. parvulus	0–7	34–36	8	30–40	4	100	7	6	0
P. pentosaceus	5–35	0–18	6	7–8	4	7	100	88–97	0
P. pentosaceus subsp. intermedius	17–19	0–7	5	6–7	3	6	88–97	100	0
P. urinae-equi	0	0	0	0	0	0	0	0	100

Data from Back and Stackebrandt (1978), Dellaglio et al. (1981), and Dellaglio and Torriani (1986).

allowed taxonomic assessments to be made with more conviction than analyses of 16S rRNA sequences alone (Dobson et al., 2002). These methods also allowed the authors to describe a new species, i.e., *P. claussenii*, which grouped outside the other recognized species by all three phylogenetic investigation methods (Dobson et al., 2002). According to 16S rDNA, 16S-23S internally transcribed spacing region, and HSP60 sequence analyses, *P. claussenii* is more closely related to *P. acidilactici* and *P. pentosaceus* than to *P. damnosus*, *P. parvulus* and *P. inopinatus* (Dobson et al., 2002). As mentioned above, *P. halophilus* (now *Tetragenococcus halophilus*), *P. urinae-equi* and *P. dextrinicus* are phylogenetically not closely related to the other *Pediococcus* species (Fig. 2). Satokari et al. (2000) showed that the reference strains of the species *P. acidilactici*, *P. damnosus*, *P. dextrinicus*, *P. inopinatus*, *P. parvulus* and *P. pentosaceus* could also be easily distinguished using automated ribotyping and that *Pediococcus* isolates could be identified on the basis of the riboprints. Moreover, they found one strain that did not group with the recognized *Pediococcus* species and postulated that this strain may represent a novel species (Satokari et al., 2000). This presumed novel species has however not been formally proposed.

Among the pediococci, *P. acidilactici* strains have been well investigated and were found to be genetically heterogeneous (Mora et al., 2000; Simpson et al., 2002). Simpson et al. (2002) used random amplified polymorphic DNA (RAPD) analysis and pulsed-field gel electrophoresis (PFGE) fingerprinting to show that with both methods, *P. acidilactici* strains fall into one of two genomic subgroups. Mora et al. (2000) used a multilocus typing approach to investigate pediocin-producing and non-pediocin-producing strains of *P. acidilactici*. Their analysis of the 16S rDNA together with restriction fragment length polymorphism (RFLP) analysis of housekeeping genes (such as the genes for lactate dehydrogenases [*ldh*D and *ldh*L], malolactic enzyme [*mle*f], and DNA-dependent RNA-polymerase [*rpo*C]) showed that seven distinct genomic subgroups could be defined. One of these contained the pediocin-producing strains, indicating that these represented a homogeneous subpopulation (Mora et al., 2000). Furthermore, the authors could correlate the genomic subgroups with the phenotype and thus speculated that the strains able to utilize specific carbohydrates or to produce pediocin had a strong competitive advantage. This would have led the genome of these strains and the genome of strains without such metabolic features down different paths of evolution, and thus to the establishment of the specific lineages or genomic subgroups presently observed (Mora et al., 2000).

Kobayashi et al. (2000) studied 413 *Tetragenococcus* strains isolated from samples obtained during the processing of Japanese puffer fish ovaries fermented with rice-bran. Their growth reactions on five representative substrates were the basis for grouping the isolates into seven groups. RFLP analysis of the 16S rRNA gene of representative strains of these major groups revealed that they could be grouped into two groups: the most prominent was a halophilic lactic acid coccus identified as *Tetragenococcus halophilus*, and the other was *Tetragenococcus muriaticus* (Kobayashi et al., 2000). The major differences between the two groups were the ability to grow in medium not supplemented with NaCl, and the fermentation of L-arabinose, sucrose and D-mannitol. However, the SSU ribosomal rRNA-based tree suggests that *T. halophilus* is more closely related to *Enterococcus solitarius* than to *T. muriaticus*.

Taxonomy

The last 15 years have brought clarity to the delineation of the genus *Pediococcus* from *Aerococcus* and *Tetragenococcus* and confirmation of those six species presently considered to be typical of this genus, i.e., *P. acidilactici*, *P. pentosaceus*, *P. damnosus*, *P. parvulus*, *P. inopinatus* and *P. claussenii*. The range of DNA G+C values of the pediococci is wide (34–42%). However, as explained before, phenotypic and phylogenetic information strongly supports the suggested status of the genus *Pediococcus*, and the key characteristics shown in Table 1 are sufficient to distinguish the genera *Pediococccus*, *Tetragenococcus* and *Aerococcus*. Moreover, on this basis, the species *P. dextrinicus* appears to be an atypical pediococcus, and probably represents a new genus. It is therefore not included in this chapter together with species considered as typical of the genus *Pediococcus*. Furthermore, *Pediococcus urinae-equi* (Mees, 1934; Garvie, 1988) has been shown to belong to the genus *Aerococcus* (Simpson and Taguchi, 1995).

Common to the genera *Pediococcus* and *Tetragenococcus* is the typical cell morphology: nonmotile, spherical cells (0.5–0.8 μm), which divide in two planes at right angles to form tretrads. The cells may also occur singly or in pairs, especially during early or mid-logarithmic growth. Homofermentation is also common to both genera, but the formation of the L(+) lactate enantiomer from glucose is typical of the tetragenococci but is only found in *P. claussenii* among the pediococci. Phenotypically, the tetragenococci are distinguished from the pediococci mainly by their high salt tolerance (growth at >18% NaCl [w/v]) and ability to grow at high

pH values up to 9.0 but not at pH 5.0 (Tables 1 and 3).

Species of the Genus *Pediococcus*

The six *Pediococcus* species recognized as typical of this genus, and their typical physiological characteristics (Table 3), are presented in the chronological order of their description:

Pediococcus acidilactici (Lindner, 1887) with the type strain DSM 20284 (Garvie, 1986b; Tanasupawat et al., 1993), invalid synonyms are "*Pediococcus lindneri*," "*Pediococcus cerevisiae*," and "*Streptococcus lindneri*."

Pediococcus damnosus (Claussen, 1903), with the type strain DSM 20331 (NCDO 1832; ATCC 29358); invalid synonyms are "*Pediococcus cerevisiae*," "*Pediococcus cerevisiae* subsp. *mevalovorus*," "*Pediococcus viscosus*," "*Pediococcus perniciosus*," "*Pediococcus sarcinaeformis*," "*Pediococcus odoris mellisimilis*," "*Pediococcus mevalovorus*," "*Streptococcus damnosus*," "*Streptococcus damnosus* subsp. *diastaticus*"; the subspecies var. "damnosus," var. "diastaticus" and var. limosus' were proposed by Coster and White (1964), but were not officially acknowledged.

Pediococcus pentosaceus (Mees, 1934), with type strain DSM 20336 (ATCC 33161; NCDO 990); invalid synonyms are "*Pediococcus hennebergi*," "*Pediococcus citrovorum*," "*Pediococcus cerevisiae*," "*Pediococcus acidilacti*" and "*Streptococcus acidilactici*"; for *P. pentosaceus* strains unable to ferment some pentoses the subspecies '*intermedius*' was proposed by Back (1978b) on the basis of the inability to ferment pentoses such as ribose, arabinose or xylose; reference to *P. pentosaceus* should therefore take account of these phenotypic differences by which the former subspecies is distinguished from *P. pentosaceus* subsp. *pentosaceus*.

Pediococcus parvulus (Günther and White, 1961b), with type strain DSM 20332 (ATCC 19371; NCDO 1634).

Pediococcus inopinatus (Back, 1987a), with type strain DSM 20285 (ATCC 49902, NCIMB 12564), with "*Pediococcus cerevisiae*" as an invalid synonym.

Pediococcus claussenii (Dobson et al., 2002), with type strain DSM 14800 (ATCC BAA-344, KCTC 3811).

Species of the Genus *Tetragenococcus*

The genus *Tetragenococcus* consists of only two species, *Tetragenococcus halophilus* (Collins et al., 1990) and *Tetragenococcus muriaticus* (Satomi et al., 1997). The species *T. halophilus* was renamed from *Pediococcus halophilus* (Collins et al., 1990) and was shown to be synonymous to "*Pediococcus soyae*" (Weiss, 1992). The name "*Tetragenococcus halophila*," recorded in the Validation List No. 49 (Anon., 1994), was later rejected and corrected to *T. halophilus* (Euzéby and Kudo, 2001). The description of the genus is based on the description of *P. halophilus* (Garvie, 1986) and *Tetragenococcus* gen. nov. (Collins et al., 1990).

Morphologically, members of the genus *Tetragenococcus* cannot be distinguished from the pediococci. Also, apart from high salt tolerance and acid sensitivity of the tetragenococci, most physiological features are common to both genera (e.g., facultative aerobic nature, Lys-D-Asp type of peptidoglycan, and the fermentation of a relatively wide range of sugars, some of which are typical only of some species). The homofermentative production of L(+) lactic acid from glucose, considered to be typical of *Tetragenococcus* and not the pediococci, however, has been shown to be a feature also of *P. claussenii*. By contrast, DL lactic acid is produced by all other typical *Pediococcus* species.

Up to 1997, only one *Tetragenococcus* species (*T. halophilus*) was recognized. The second species, *T. muriaticus*, was recently described. It is also halophilic and even requires NaCl for growth. So far, it has only been associated with Japanese fermented fish sauce (Satomi et al., 1997) and puffer fish ovaries fermented with rice-bran (Kobayashi et al., 2000).

Habitats

As a result of their halotolerant to halophilic nature, the tetragenococci are generally associated with quite different habitats than the pediococci. *Pediococcus* spp., on the other hand, share common habitats with representatives of *Lactobacillus*, *Leuconostoc* and *Weissella* spp. Strains of *P. acidilactici* and *P. pentosaceus* occur on a great variety of plants and fruits, although only in small numbers in the "natural" situation (Mundt et al., 1969; Back, 1978b; Dellaglio et al., 1981; Wilderdyke et al., 2004). In the fermentation of plant materials, such as silage, cucumbers, olives, or cereal gruels, and also during meat fermentations, the pediococci often multiply rapidly, and may become a major component of the lactic acid bacterial population in interaction and association with members of the genera *Lactobacillus*, *Leuconostoc* and *Weissella*.

Reports on the occurrence of pediococci and tetragenococci on natural habitats other than plants are rare. Such reports, however, indicate the association of some pediococci with human and animal hosts. Sims (1986) reported the presence of pediococci in human saliva. Although not a major group, pediococci also seem to be

Table 3. Phenotypic characteristics of Pediococcus and Tetragenococcus spp.[a]

Characteristics	P. acidilactici	P. pentosaceus[b]	P. claussenii[c]	P. damnosus	P. inopinatus	P. parvulus	T. halophilus	T. muriaticus
Acid from								
Amygdalin	+	+	+	d	+	d	+	ND
Arabinose	d	+ or −[*]	−	−	−	−	d	−
Cellobiose	+	+	+	+	+	+	+	ND
Dextrin	−	−	−	−	d	d	−	−
Fructose	+	+	+	+	+	+	+	+
Galactose	+	+	−	+	+	d	d	ND
Glucose	+	d	+	+	+	+	+	+
Glycerol	d	d	−	−	−	−	d	−
Inulin	−	−	−	−	+	−	−	ND
Lactose	d	+	−	−	+	−	−	−d
Maltose	−	+	d	d	+	d	+	+
Mannitol	−	−	d	−	−	−	d	
Mannose	+	+	+	+	+	+	+	+
Melezitose	−	−	−	d	−	−	+	−
Melibiose	−	d	−	−	−	−	d	ND
Maltotriose							+	−
α-Methyl-glucoside	−	−	−	d	d	−	+	D
Raffinose	d	d	−	−	−	−	d	−
Rhamnose	d	d	−	−	−	−	−	−
Ribose	+	+	+	−	−	−	+	+
Salicin	d	d	+	d	+	+	+	ND
Sucrose	−	d	−	d	d	−	+	−
Trehalose	d	+	+	d	+	+	d	+
Xylose	+	d	−	−	−	−	+	−
Ammonia from arginine	+	+	ND	−	−	−	−	−
Hydrolysis of esculin	+	+	+	d	+	+	ND	ND
Lactic acid configuration	DL	DL	L(+)[**]	DL	DL	DL	L(+)	L(+)
Dextran production	−	d	D	d	−	−	ND	ND
Histamine formation	ND	ND	ND	ND	ND	ND	−	+
Growth at pH 4.5	+	+	+[**]	+	+	+	−	−
pH 7.0	+	+	+[**]	−	d	d	+	+
pH 8.0	+	+	+[**]	−	−	−	+	+
35°C	+	+		−	+	+	+	+
40°C	+	d	+[**]	−	d	−	+	−

associated with the digestive tract of man and animals, and Ruoff et al. (1988) reported their presence in human feces. *Pediococcus* spp. were detected (together with *Lactobacillus*, *Leuconostoc* and *Weissella* spp.) in human feces by using group-specific polymerase chain reaction (PCR) primers and denaturing gradient gel electrophoresis (DGGE) of DNA fragments generated by PCR with 16S ribosomal DNA-targeted group-specific primers (Hertel et al., 2001). Using specific amplification of 16S ribosomal DNA, the presence of pediococci in the human intestines together with lactobacilli and other LAB was confirmed by Heilig et al. (2002). *Pediococcus acidilactici* and *P. pentosaceus* were detected in the gastrointestinal (GI) tracts of poultry (Juven et al., 1991) and ducks (Kurzak et al., 1998), while *P. acidilactici* and *P. parvulus* were also reported to be in the feces of turkeys (Harrison and Hansen, 1950; Coster and White, 1964), and *P. acidilactici* in the intestines of the common carp (*Cyprinus carpio*) and freshwater prawns (*Macrobrachium rosenbergii*) in Thailand (Cai et al., 1999). Moreover, the pediococci, and particularly *P. pentosaceus*, have been shown to form part of the Gram-positive tonsillar and nasal microbial population of piglets (Baele et al., 2001; Martel et al., 2003).

The association of pediococci with the clinical environment and their possible role in pathogenesis are discussed in the section Epidemiology and Disease. Generally, the pediococci are not typical pathogens, and the possible interactive association of some strains with infection may be the result of an opportunistic situation, often also connected with their intrinsic resistance to antibiotics such as vancomycin (Riebel and Washington, 1990; Edlund et al., 1997).

Literature on the typical habitats of pediococci and tetragenococci mainly refer to different food systems. This is also reflected in the literature on pediococci summarized in Table 4.

Genus *Pediococcus*

The importance of pediococci is reflected not only by their positive role in fermentation of many foods, but also by undesired interactions in spoilage of some commodities, most notably beer. A summary of the biotechnical applications is presented in Table 5 and the Biotechnical Applications section.

PLANT MATERIALS. Although only present in small numbers in natural plant habitats together with other LAB, the pediococci often participate in larger numbers in the fermentation of numerous plant materials. Such associations have been reported for *Pediococcus acidilactici*, *P. pentosaceus*, *P. parvulus*, *P. inopinatus* and "*P. dextrinicus*" and to be involved in the fermentation of silage, sauerkraut, fermented beans, cucumbers, olives, and cereal gruels (Langston and Bouma, 1960; Günther and White, 1961b; Günther and White, 1962; Coster and White, 1964; Mundt et al., 1969; Stamer, 1975; Etchells et al., 1975; Back, 1978b; Dellaglio et al., 1981; Costilow and Gerhardt, 1983; Lin et al., 1992; Wilderdyke et al., 2004). The association of *P. damnosus* and *P. claussenii* with beer spoilage, resulting from their relatively high resistance to hops, will be discussed below.

Even when pediococci more typically form an intermediate group among the LAB involved in the fermentation of most plant foods, the selection of strains of particularly *P. acidilactici* as starters for vegetable and fruit juice fermentations (Knorr, 1998) and of both *Pediococcus acidilactici* and *P. pentosaceus* for the control of silage fermentations (Fitzsimons et al., 1992; Cai et al., 1999; Kung and Ranjit, 2001; Steidlova and Kalac, 2003) has been suggested (Table 5).

The association of pediococci with several traditional fermented cereal foods has been reported frequently. The probiotic potential of koko and koko sour water, respectively an African spontaneously fermented millet porridge and drink, has been studied by Lei and Jakobsen (2004), and the typical bacteria in these commodities were, in addition to *P. pentosaceus* and *P. acidilactici*, *Weissella confusa* and *Lactobacillus fermentum*. Back (1978b) has reported *P. acidilactici* and *P. pentosaceus* to be involved in the fermentation of different grains, malt and wort. Different fermented cereals form an important part of the daily diet in many regions of Africa. Fermented sorghum-based foods seem of particular interest, e.g., as relatively safe and wholesome weaning foods in Southern Africa (Kunene et al., 2000). "Togwa," a traditional Tanzanian food (a mixture of spontaneously fermented sorghum, maize, millet and maize-sorghum) was found to contain *P. pentosaceus* as well as other LAB and yeasts (Mugula et al., 2003b; Mugula et al., 2003c). This species has also been reported as part of the microbial population of traditional fermented bread (khamir) produced from sorghum in the Gizan region, Saudi Arabia (Gassem, 1999). Pediococci also constitute an important part of the microbial population found in hussuwa, a traditional fermented sorghum product of the Sudan (Yousif et al., 2004).

Interesting examples of mixed-culture dough inocula prepared either in the form of dried powders, flat cakes, or hard balls, are found in several Asian countries where they are used for the inoculation of starchy substrates for the production of alcoholic beverages. The names of these inocula depend on the location of production. Marcha (murcha), bakhar, or phab is produced in the Himalayan regions of India, Nepal, Tibet and

Table 4. Habitats of pediococci.

P. acidilactici	*P. pentosaceus*	*P. inopinatus*	*P. parvulus*	*P. damnosus*	*P. claussenii*	*Pediococcus* spp.
Large variety of plants and fruits (Mundt et al., 1969; Back, 1978a; Wilderdyke et al., 2004); vegetable fermentations (Back, 1978a; Tanasupawat and Komagata, 1995; Yousif et al., 2004); fermenting plant materials (Mundt et al., 1969); fermented brined cucumber and green beans (Costilow and Gerhardt, 1983); silages (Langston and Bouma, 1960; Lin et al., 1992; Ennahar et al., 2003); semi-dry sausages (Deibel et al., 1961; Everson et al., 1970; Porubcan and Sellars, 1979); malt mash (Lindner, 1887); grains, malt, wort (Back, 1978a); "*koko*" and "*koko sour*" (Ghana; Lei and Jakobsen, 2004); sorghum-based fermented weaning food (Kunene et al., 2000); different fermented fish products (Blood, 1975; Tanasupawat and Daengsubha, 1983; Paludan-Muller, 2002); Thai fermented foods (Smitinont et al., 1999); nonfermented Malaysian food condiment chili bo (Leisner et al., 1999); GI tracts of poultry (Juven et al., 1991) and ducks (Kurzak et al., 1998); intestines of the common carp (*Cyprinus carpio*) and freshwater prawns (*Macrobrachium rosenbergii*) in Thailand (Cai et al., 1999)	Large variety of plants and fruits (Mundt et al., 1969; Back, 1978a; Dellaglio et al., 1981); fermenting vegetables (Back, 1978a; Tanasupawat and Komagata, 1995; Yousif et al., 2004); silages (Whittenbury, 1965; Langston and Bouma, 1960; Lin et al., 1992; Ennahar et al., 2003); olives and sauerkraut (Stamer, 1975); pickled cucumbers (Etchells et al., 1975); fermented brined cucumber and green beans (Costilow and Gerhardt, 1983); wine (Eliseevsa et al., 2001; Rodas et al., 2003); grain, malt, wort (Back, 1978a); "*koko*" and "*koko sour*" (Back, 1978a); "*koko sour*" (Ghana; Lei and Jakobsen, 2004); "togwa" (fermented sorghum product, Tanzania; Mugula et al., 2003a, b); sorghum-based fermented weaning food (Kunene et al., 2000); fermented sorghum bread (Gassem, 1999); fermented sausages (Deibel et al., 1961; Porubcan and Sellars, 1979; Gevers et al., 2000; Parente et al., 2001); Thai fermented fish products (Paludan-Muller, 2002); Thai fermented foods (Smitinont et al., 1999); cheese (Naylor and Sharpe, 1958; Callon et al., 2004); brewer's yeast, mash, dairy products, fermenting vegetables (Nakagawa and Kitahara, 1959); brewer's yeast (subsp. *intermedius*; Back, 1978a); human GI tract (feces; Walter et al., 2001); feces of turkeys (Harrison and Hansen, 1950); GI tracts of poultry (Juven et al., 1991); and ducks (Kurzak et al., 1998)	Fermenting vegetables, fermented beans, sauerkraut (Back, 1978a); beer, brewer's yeast (Back, 1978a); spoiled cider (Carr, 1970); and wine (Edwards and Jensen, 1992; Back, 1978a; Weiller and Radler, 1970)	Fermenting vegetables, sauerkraut, fermented beans (Coster and White, 1964; Back, 1978a); silages, milk, cheese, grass (Langston and Bouma, 1960; Günther and White, 1961, 1962; Coster and White, 1964); feces of turkeys (Coster and White, 1964); and wine (Nonomura et al., 1967; Edwards and Jensen, 1992; Rodas et al., 2003)	Beer (Balcke, 1884) brewer's yeast, green beer, wine, wine yeast (Back, 1978a, 1994, 2000); brewer's yeast, beer (including subsp. *mevalovorus*; Nakagawa and Kitahara, 1959); beer (Mees, 1934); wort, beer (Shimwell, 1949); beer (*P. damnosus* var. *diastaticus*; Andrews and Gilliland, 1952); brewer's yeast, beer (Coster and White, 1964); and wine (Peynaud and Domercq, 1967; Carr, 1968; Weiller and Radler, 1970; Beneduce et al., 2004)	Beer, brewer's yeast (Dobson et al., 2002)	Thai fermented foods (Tanasupawat and Daengsubha, 1983); wine must (Lonvaud-Funel, 1999); Rioja red wines (Navarro et al., 2000); cheese (Dacre, 1958; Gelsomino et al., 2001); fermented cassava dough (Ben Omar et al., 2000; Miambi et al., 2003); seed-based fermented foods (Ariahu et al., 1999); fresh and cured meat (Reuter, 1970; Jones, 2004); fermented sausages. "chorizo" (Reuter, 1970; Santos et al., 1997; fresh and marinated fish (Blood, 1975); vacuum-packaged vienna sausages (Franz and von Holy, 1996); vacuum-packaged beef (Jones, 2004); human intestines (Heilig et al., 2002); human saliva (Sims, 1986); human feces (Ruoff et al., 1988); GI tract of ducks (Kurzak et al., 1998); and nasal and tonsillar flora of piglets (Martel et al., 2003)

Table 5. Biotechnical applications of pediococci.

P. acidilactici	P. pentosaceus	P. inopinatus	P. parvulus	Pediococcus spp.
Control of silage fermentation (Fitzsimons et al., 1992; Cai et al., 1999); vegetable fermentations (Knorr, 1998); preservation of meat (Luchansky et al., 1992; Mattila-Sandholm et al., 1993) and plant food (Gibbs, 1987); fruit juices (Knorr, 1998); dough (Nigatu et al., 1998); starters for raw sausages (Raccach, 1987; Kang and Fung, 1999); hot acidification process for "yoghurt" manufacture, for sour milk ("Dickmilch"), and quarg (Back, 2000); cheese (Litopoulou-Tzanektaki et al., 1989; Bhowmik and Marth, 1990); acidification of malt, mash, wort (in brewery; Back, 1994, 2000); and probiotic feeds (Van Belle, 1990; Tannock, 1997; Brashears et al., 2003)	Fermentation of soy milk (Raccach 1987); preservation of meat and plant food (Gibbs, 1987) starters for raw sausages (Raccach, 1987; Holley and Blaszyk, 1997; Waade and Stahnke, 1997); protective cultures for pork liver pate (Moore and Madden, 1997); starters for "Dickmilch," "Longfil (Back, 2000); acidification of malt, mash, and wort (in brewery; Back, 1994, 2000); togwa fermentation (Tanzanian maize-sorghum gruel; Mugula et al., 2003c); barley silage (Kung and Ranjit, 2001); and maize silage (Steidlova and Kalac, 2003)	Acidification of vegetables, sauerkraut, beans, etc. (Back, 1978a)	Silage fermentation (Günther and White, 1961, 1962; sauerkraut (Coster and White, 1964); and fermentation of vegetables and production of sauerkraut (Back, 1978a)	Probiotics for humans, bioprotectives, and probiotics for fermented sausages (Tyopponen et al., 2003); and probiotics in animal nutrition (Guillot, 1997; Leuschner et al., 2003; Weese and Areoyo, 2003)

Bhutan; ragi is produced in Indonesia, nuruk in Korea, bubo in the Philippines, chiu-yueh (chiu nang or lao chao) in China, loogpang in Thailand (Tamang, 1998), and banh men in Vietnam (Lee and Fujio, 1999). These "ragi type" starters contain mixed cultures of filamentous fungi (e.g., *Amylomyces*, *Mucor*, *Rhizopus* and *Actinomucor*), yeasts (*Saccharomyces* spp., *Pichia* spp., and *Hansenula* spp.), and LAB (species of *Lactobacillus* and *Pediococcus*; Tamang, 1998).

The association of *P. damnosus* with beer spoilage was first reported by Balcke (1884). This species is commonly isolated from beer (Mees, 1934; Shimwell, 1949; Andrews and Gilliland, 1952; Nakagawa and Kitahara, 1959; Coster and White, 1964; Back, 1978b; Back, 1994; Back, 2000). "*Pediococcus dextrinicus*" and *P. inopinatus* have been isolated occasionally from beer but with lower frequency (Back, 1978b; Back, 1994; Back, 2000); as explained (see above), "*Pediococcus dextrinicus*" is not considered as typical *Pediococcus* because of its phylogeny and its atypical characteristics (starch fermentation and formation of CO_2 from gluconate; Collins et al., 1990; Stiles and Holzapfel, 1997). *Pediococcus acidilactici*, *P. pentosaceus* and *P. parvulus* (Barney et al., 2001) are also associated with the brewery environment but are mainly found on malt and can also grow during early stages of wort production (before addition of hops) and at temperatures <50°C. Some strains of these species and especially of "*Pediococcus dextrinicus*" and *P. inopinatus* may grow or survive only in presence of low alcohol concentrations, elevated pH values, and especially low content or absence of hops bitter acids (humulon and lupulon; Back, 1978b; Back, 1994; Lawrence and Priest, 1981). Mechanisms responsible for the resistance to hops have been described by Sakamoto and Konings (2003). A rapid, noninvasive method for detection of hops resistant beer spoilage LAB strains has been developed by Yansanjav et al. (2004). A mixture of tetrahydro*iso*-α-acids ("Tetra") is used in growth experiments during which the intracellular pH is measured. Beer isolates of *P. inopinatus* showed pronounced decrease in intracellular pH during exposure to Tetra, thereby explaining their reduced ability to grow in beers, as compared to resistant strains of *Lb. brevis* (Yansanjav et al., 2004). The antibacterial activity of these hops compounds against sensitive Gram-positive bacteria was shown to be related to their action as ionophores, which dissipate the pH gradient across the cytoplasmic membrane and reduce the proton motive force (Sakamoto and Konings, 2003).

Spoilage of beer by *Pediococcus damnosus* is associated with cloudiness and other faults, particularly an off-taste and adverse flavor caused by diacetyl formation (Weiss, 1992; Donhauser,

1993; Sakamoto and Konings, 2003). An extracellular polymer may also be formed and result in ropy consistency (Simpson and Taguchi, 1995). The only other *Pediococcus* sp. with resistance to hops is the recently described *Pediococcus claussenii*, which has thus far only been found in association with the brewery environment and beer spoilage (Dobson et al., 2002). The production of an exopolysaccharide has also been described for strains of *P. claussenii*, a property that may be lost upon repeated culture (Dobson et al., 2002).

The role of *Pediococcus* spp. in the production of tyramine has been studied under different conditions. *Pediococcus* spp. are able to form tyramine during beer fermentation, and the quantity of tyramine produced depends on the degree of contamination (Izquierdo-Pulido et al., 1997). On the other hand, in a study of biogenic amine formation in bottled beer, Kalač et al. (2002) have shown that lactobacilli are much more effective amine producers than pediococci. The tyramine and histamine levels can increase considerably in insufficiently pasteurized bottled beers.

The requirement of mevalonic acid has been reported for some strains of *P. damnosus*; this substance is produced during the fermentation process and thereby favors their growth in beer rather than in wort (Kitahara and Nakagawa, 1958; Simpson and Taguchi, 1995). In modern brewing, spoilage problems with pediococci have been overcome mainly by advanced contamination control measures (Weiss, 1992) and the strict application of Hazard Analysis and Critical Control Point principles.

Some leuconostocs, lactobacilli and pediococci are associated with the early stages of fermenting grape must (juice). This has particularly been shown for *P. damnosus* with regard to wine and wine yeast (Peynaud and Domercq, 1967; Carr, 1968; Weiller and Radler, 1970; Back, 1978b; Back, 1994; Back, 2000; Beneduce et al., 2004), in which the production of diacetyl and acetoin and also the formation of slime (a D-glucan polymer) have been reported. Especially at higher pH values, strains of *P. parvulus* may grow in wine. Pediococci have also been associated with a bitter taste in some red wines. In a study of wine-associated LAB, Osborne et al. (2000) have found nine strains of the genera *Lactobacillus* and *Oenococcus* that were able to metabolize acetaldehyde in a resting cell system, whereas two *Pediococcus* strains did not. As a result of relatively common amino decarboxylase activity (Bover-Cid and Holzapfel, 1999), pediococci have also been associated with higher levels of biogenic amines in wine (Weiller and Radler, 1976). The formation of histamine in wines was reported to be due mainly to *Pediococcus* spp. (Aerny, 1985; Delfini, 1989). On the other hand,

the *Pediococcus* strain isolated from wine and studied by Victoria Moreno-Arribas et al. (2003) did not show any potential for the decarboxylation of histidine or tyrosine, although arginine was decarboxylated to putrescine.

FOODS OF ANIMAL ORIGIN. The association of pediococci with proteinaceous foods such as fresh and cured meat, and raw sausages has frequently been reported (Reuter, 1970), and particularly for *Pediococcus acidilactici* (Deibel et al., 1961; Everson et al., 1970; Porubcan and Sellars, 1979) and *P. pentosaceus* in fermented sausages (Deibel et al., 1961; Porubcan and Sellars, 1979; Gevers et al., 2000; Parente et al., 2001). Both these species have also been reported for fresh and marinated fish (Blood, 1975; Tanasupawat and Daengsubha, 1983; Paludan-Muller, 2002), for different Thai fermented foods (Smitinont et al., 1999), and also for a nonfermented Malaysian food condiment "chili bo" (Leisner et al., 1999). Moreover, *P. pentosaceus* seems to play some role in the fermentation and at least the maturation of cheese (Dacre, 1958; Naylor and Sharpe, 1958; Callon et al., 2004).

Meat and meat products provide a favorable growth substrate for strains of *Pediococcus acidilactici* and *P. pentosaceus*, and particularly in the fermentation of semi-dry sausages or other cured products, such strains appear to play some role during fermentation and maturation (Deibel et al., 1961; Everson et al., 1970; Porubcan and Sellars, 1979; Gevers et al., 2000; Parente et al., 2001). *Pediococcus pentosaceus* was reported for Spanish dry-cured ham (Molina et al., 1989). Pediococci are also frequently found in vacuum- or modified-atmosphere-packaged meat and meat products, in which the LAB population is, however, most often dominated by species of the genera *Lactobacillus*, *Leuconostoc*, *Carnobacterium*, *Weissella and Enterococcus* (Reuter, 1970; Reuter, 1975; Dykes et al., 1994; Jones, 2004).

The association of pediococci with meat fermentations has been a topic of intensive study (Holzapfel, 1998). Knowledge about the enzymatic degradation of meat proteins by LAB is still sparse. Lipolytic activity was reported for *P. pentosaceus* strains isolated from dry-cured ham (Nieto et al., 1989; Molina et al., 1991). Extracellular protein systems of lactobacilli have been poorly characterized thus far (Hammes et al., 1992), and the proteolytic activity of LAB generally appears to be very weak (Law and Kolstad, 1983). No endopeptidase activity was found in strains of *P. pentosaceus* isolated from cured ham, although *P. pentosaceus* strains showed strong leucine and valine arylamidase activities (Molina and Toldra, 1992). Diacetyl may be produced by some *Pediococcus* spp. in meat, e.g., as a result of citric acid degradation. Because of its intensive

aroma, diacetyl may cause off-flavors even at low concentrations; it has, however, little direct consequence for antimicrobial actions in meat systems (Holzapfel, 1998).

The microbiology and safety of traditional fermented ("dry") sausages from different regions have received increased research attention in recent years, also against the background of the formation of biogenic amines by amino acid decarboxylase activity of some LAB, including pediococci. The production of tyramine is commonly associated with the fermentation (particularly the maturation of traditional fermented sausages) and related to *P. cerevisiae* (and probably *P. pentosaceus*; Rice and Koehler, 1976). On the other hand, the use of starter cultures such as selected strains of *P. pensoaceus* resulted in lower amounts of tyramine, putrescine and cadaverine (Hernandez-Jover et al., 1997).

Genus *Tetragenococcus*

The salt-tolerance of the two recognized species of *Tetragenococcus* determines to a large extent their occurrence and domination in a number of man-made habitats and particularly in food processes in which high salt concentrations (i.e., 12–26%) prevail (Röng et al., 1996). They form part of the fortuitous microbial population established in these habitats (Gürtler et al., 1998).

Both *T. halophilus* and *T. muriaticus* are found in habitats rich in salt and protein. Typical habitats of *T. halophilus* are anchovy pickles (Orla-Jensen, 1919), salted anchovies (Villar et al., 1985), fermented fish, traditional in Thailand (Tanasupawat and Daengsubha, 1983), soy sauce mashes, both of the Japanese moromi and the Indonesian baceman type (Nakagawa and Kitahara, 1959; Sakaguchi, 1960; Sakaguchi and Mori, 1969; Röng and Van Versefeld, 1996). The Japanese mashes differ from the Indonesian types in that the Indonesian process uses soybeans only, as opposed to equal amounts of soybean and wheat used in Japan (Röng and Van Versefeld, 1996; Gürtler et al., 1998). Selected strains are used for inoculation of mashes for moromi fermentation in soy sauce production (Simpson and Taguchi, 1995). *Tetragenococcus halophilus* is also found in pickling brines and in association with the maturing of dry-cured ham (Back, 1978b). A natural habitat of *T. halophilus* appears to be pig feces (Simpson and Taguchi, 1995).

Strains of moderately halophilic histamine-producing bacteria (isolated from fermented Shottsuru-like squid liver sauce and considered members of the genus *Tetragenococcus* on the basis of their physiological, morphological and chemotaxonomic characteristics) were found to

differ from *T. halophilus*. For these strains, Satomi et al. (1997) suggested the name "*Tetragenococcus muriaticus*." Some strains (formerly identified as *T. halophilus*; Ito et al., 1985, cf. Satomi et al., 1997) isolated by previous workers from shottsuru, a Japanese local traditional fermented fish sauce containing >25% NaCl, are probably representatives of *T. muriaticus*. Strains of *T. muriaticus* were also isolated together with *T. halophilus* from the manufacturing process of Japanese puffer fish ovaries fermented with rice-bran (Kobayashi et al., 2000). An interesting difference between these two species was found in the inability of *T. muriaticus* strain to grow in media not supplemented with NaCl (Kobayashi et al., 2000).

ENUMERATION AND ISOLATION Representatives of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Weissella* (the so-called "LLPW group") share numerous physiological features, and therefore they frequently respond in similar fashion to conditions or factors inhibitory to the growth of non-lactic acid bacteria (non-LAB). This explains why most culture media developed for the detection of *Pediococcus*, *Lactobacillus*, *Weissella* or *Leuconostoc* are rather elective but not fully selective for the particular genus. This is reflected by the composition and mode of application of most media developed for detection and isolation of the pediococci (Schillinger and Holzapfel, 2003).

Knowledge of the physiology and typical environmental conditions favoring growth of specific strains are important prerequisites for the successful detection and isolation of pediococci. The selective detection of pediococci becomes particularly complicated in habitats where they are typically associated with mixed populations of LAB, e.g., in natural and artificial plant and food environments, and especially fermented foods. The complex growth requirements and general physiological features of most pediococci are comparable to those of the lactobacilli, leuconostocs and weissellas and, in part at least, also other LAB such as the enterococci. These aspects explain the difficulty of obtaining pure *Pediococcus* cultures in a one-step selective operation. Exceptions to these generalizations are the detection and isolation of pediococci from beer, where the elevated resistance to hops represents an important selection factor for isolation and detection of *P. damnosus* and *P. claussenii*.

The high salt resistance of the tetragenococci and their tolerance to high pH values (around 9.0) are two major factors by which they may be selectively detected and isolated from food fermentation products containing high salt concentrations, e.g., soy mashes, fish sauces, etc.

The Genus *Pediococcus*

ELECTIVE MEDIA. General purpose media for the elective detection of *Pediococcus*, *Lactobacillus*, *Weissella* and *Leuconostoc* that are most frequently used, include MRS medium (de Main et al., 1960), YGP medium (Garvie, 1978), and TGE medium (Biswas et al., 1991). APT (Evans and Niven, 1951) may also be used for isolation of pediococci, but its applicability is lower than that of MRS and may be less effective under conditions where leuconostocs predominate. More details on these and other elective and semiselective media are discussed by Schillinger and Holzapfel (2003). The application of some of the most important media is discussed below.

MRS Agar for Isolating and Propagating Pediococci (de Man et al., 1960)

Oxoid peptone	10.0 g
Meat extract	10.0 g
Yeast extract	5.0 g
KH_2PO_4	2.0 g
Diammonium citrate	2.0 g
Glucose	20.0 g
Tween 80	1.0 ml
Na acetate $\cdot 3H_2O$	5.0 g
$MgSO_4 \cdot 7H_2O$	0.50 g
$MnSO_4 \cdot H_2O$	0.25 g
Distilled water	1 liter

For 1 liter of medium, dissolve 15 g of agar in distilled water by steaming. Add the other ingredients and adjust pH to 6.2–6.4. Sterilize at 121°C for 15 min.

Glucose-Yeast Extract Agar for Isolation of *Leuconostoc* and *Pediococcus* (Whittenbury, 1965a)

Glucose	5.0 g
Yeast extract	5.0 g
Peptone	5.0 g
Meat extract	5.0 g
Agar	15.0 g

Dissolve the medium components in distilled water, q.s. up to 1 liter, adjust pH to 6.5, and autoclave at 121°C for 15 min.

Under particular conditions, elective or semiselective enrichment may allow the qualitative detection of particular *Pediococcus* species, e.g., by relying on the fermentation of specific sugars such as melezitose, ribose or dextrin as carbon sources, or by incubation at 50°C (Back, 1978b).

SELECTIVE AND SEMISELECTIVE MEDIA. These media are used for general enumeration and isolation.

Selective SL Medium for Isolating Pediococci (Rogosa et al., 1951)

Trypticase	10.0 g
Yeast extract	5.0 g
KH_2PO_4	6.0 g
Diammonium citrate	2.0 g
$MgSO_4 \cdot 7H_2O$	0.58 g

MnSO$_4$ · H$_2$O	0.15 g
FeSO$_4$ · 7H$_2$O	0.03 g
Glucose	20.0 g
Tween 80	1.0 ml
Na acetate · 3H$_2$O	25.0 g
Agar	15.0 g

Dissolve the agar in 500 ml of distilled water by boiling. Dissolve all other ingredients in 500 ml of distilled water, adjust pH to 5.4 using glacial acetic acid, and mix with the melted agar. Boil for a further 5 min and pour plates or distribute the hot medium in convenient amounts in sterile screw-capped bottles; no further sterilization is necessary. Avoid repeated melting and cooling.

Acetate Agar for Isolation of Leuconostoc and Pediococcus (Whittenbury, 1965)

Meat extract	50.0 g
Peptone	5.0 g
Yeast extract	5.0 g
Glucose	10.0 g
Tween 80	0.5 ml
Tap water	900 ml

Adjust the pH to 5.4. Autoclave the medium at 121°C for 15 min. Before plating, add 100 ml of sterile 2M acetic acid-sodium acetate buffer (pH 5.4). This agar (a modification of the medium proposed by Keddie, 1951) is selective for lactobacilli.

ENUMERATION OF PROBIOTIC PEDIOCOCCI IN ANIMAL FEED An enumeration method to be used as official control under Council Directive 70/524/EEC for probiotic pediococci used as feed additives, was validated by 17 laboratories in 11 European countries, and considered for adoption by the Comite Europeen de Normalisation (CEN) and International Organization of Standardization (ISO; Leuschner et al., 2003). Of the four different agars compared (i.e., MRS, acidified MRS [pH adjusted to 5.4 ± 0.1 with 1M HCl], MRS with 0.01% of triphenyl tetrazolium chloride [TTC], and a newly developed pediococci selective medium [PSM]), MRS agar showed the best results, followed by acidified MRS and MRS + TTC agar. The PSM was selective for pediococci and can be used if the concentration of this species is more than tenfold lower than that of the other species present capable of growth on MRS agars. This medium was prepared by supplementing MRS agar with 0.05% (w/v) cysteine hydrochloride (Sigma C-1276, Poole, Dorset, United Kingdom), vancomycin (10 µg/ml), novobiocin (0.1 µg/ml) and nystatin (50 U/ml). The antibiotic supplements were added from fresh stock solutions after autoclaving of the basal MRS (containing cysteine hydrochloride) and equilibration at 48°C (Leuschner et al., 2003).

ISOLATION AND DETECTION FROM BEER AND THE BEER BREWING ENVIRONMENT Several media have been developed for the semiselective and selective detection of pediococci in beer. Pediococci from beer or brewery habitats can be isolated on MRS agar adjusted to pH 5.5, while a 1:1 mixture of MRS medium and beer for fastidious beer-spoiling strains is recommended by Back (1978a). Plates should be incubated in an atmosphere of 90% N$_2$ + 10% CO$_2$ at 22°C. The Kirin-Ohkochi-Taguchi (KOT medium; Taguchi et al., 1990) is quite complex, and contains beer, malt extract, liver concentrate, maltose, L-malic acid, cytidine, thymidine, actidione and sodium azide in addition to the components usually used for LAB media. Typically, beer is frequently included as a major component of detection and isolation media for pediococci (Nakagawa, 1964; Back, 1978b), to promote growth of pediococci adapted to this substrate.

Three media are recommended by the European Brewery Convention for the detection of lactobacilli and pediococci in beer: 1) MRS agar (see above) supplemented with cycloheximide to prevent growth of aerobes such as yeasts and molds, 2) Raka-Ray medium supplemented with cycloheximide, and 3) VLB (Versuchs- und Lehranstalt für Brauerei in Berlin) S7-S. As alternative media, UBA (Universal Beer Agar) supplemented with cycloheximide, HLP (Hsu's *Lactobacillus* and *Pediococcus* medium), and NBB (Nachweismedium für bierschädliche Bakterien), WLD (Wallerstein Differential), Nakagawa, SDA (Schwarz Differential Agar), and MRS modified by addition of maltose and yeast extract at pH 4.7 may also be used. According to Sakamoto (2002), none of these media is suitable for the simultaneous detection of all strains of lactobacilli and pediococci, but a combination of some of these media yields the best results.

NBB is probably the most appropriate and effective medium for detection and cultivation of beer pediococci. Growth on this medium has been at the most rapid and intensive rate recorded (Dachs, 1981). On account of the high beer-specific selectivity, yeasts and Gram-negative bacteria are inhibited (Back, 1994; Back, 2000).

Inhibition of beer yeasts can be achieved by several agents such as cycloheximide, actidione or sorbic acid. Gram-negative bacteria can be suppressed by the addition of compounds such as polymyxin B, acetic acid, and thallous acetate, and most other Gram-positive bacteria are inhibited by concentrations of hop bitter acids typically found in beer (Schillinger and Holzapfel, 2003).

Universal Beer Agar (UBA) is a semiselective medium containing beer and is used for the cultivation and enumeration of different microorganisms, including pediococci that are significant in the brewing industry (Kouzulis and Page,

1968). The addition of beer, particularly hop constituents and alcohol, eliminates many airborne contaminants while allowing beer spoilage organisms to grow. Beer supports the growth of *Lactobacillus*, *Pediococcus*, *Acetobacter* and *Zymomonas* species and wild yeast strains that may be found infecting the pitching yeast, wort or beer. The beer may also contain 0.01 g of cycloheximide per liter of medium to increase selectivity.

ENSILAGE ENRICHMENT OF LACTOCOCCI, STREPTOCOCCI, LEUCONOSTOCS, AND PEDIOCOCCI The method is as described by Whittenbury (1965) and is discussed by Björkroth and Holzapfel (2003). A modification of this method was reported by Weiler and Radler (1970), in which grape leaves are homogenized in an equal volume of acetate buffer (pH 5.4; 0.2 M). The homogenate is placed into sterile tubes, and the tubes are sealed and incubated at 30°C.

ISOLATION AND DETECTION FROM WINE Several acidic media have been employed for the isolation of *O. oeni* and of pediococci from wine (Garvie, 1967; Weiler and Radler, 1970; discussed by Björkroth and Holzapfel, 2003).

Acidic Tomato Broth (ATB) for Isolation of *Leuconostoc* and *Pediococcus* (Garvie, 1967)

Peptone	10.0 g
Yeastrel	5.0 g
Glucose	10.0 g
$MgSO_4 \cdot 7H_2O$	0.20 g
$MnSO_4 \cdot 4H_2O$	0.05 g
Tomato juice	25% (v/v)

Dissolve the medium components in distilled water and q.s. up to 1 liter. The pH is 4.8. Autoclave the medium at 121°C for 15 min. Before use, filter-sterilize a solution of cysteine hydrochloride and add to a final concentration of 0.05% (w/v).

The Genus *Tetragenococcus*

The genus *Tetragenococcus* was created from the former *Pediococcus halophilus*. It is characterized by its very high salt tolerance. Strains of *Tetragenococcus halophilus* are able to grow at a salt concentration >18% NaCl and are involved in lactic fermentations of salty foods such as soy sauce mashes, pickling brines, salted anchovies, and fermented fish sauce (shottsuru). Recently, a second species, *T. muriaticus* isolated from Japanese fermented squid liver sauce was described (Satomi et al., 1997).

Media used for the selective isolation of tetragenococci generally contain high levels of sodium chloride. MRS may be supplemented with 5% NaCl (Back, 1978) or 10% NaCl (Gürtler et al., 1998). Other media recommended for selective isolation of tetragenococci

include polypeptone-acetate-thioglycolate agar with 15% NaCl (PAT-15; Uchida, 1982), nutrient agar supplemented with 0.5% glucose, 1.2% NaCl, and salted anchovy broth (Villar et al., 1985), and yeast-glucose-peptone (YGP) medium supplemented with 5% NaCl (Collins et al., 1990) or supplemented with 10% NaCl, 1% magnesium sulfate, 0.1% potassium chloride, and 0.5% calcium carbonate (Satomi et al., 1997). For isolation of tetragenococci from soy sauce and brines, MRS agar may be adjusted to pH 7.0 and supplemented with 4–6% NaCl; cycloheximide can be added to all media to suppress yeast growth (Weiss, 1992).

NONCULTURAL AND DIRECT METHODS FOR ENUMERATION AND DETECTION Whiting et al. (1999) isolated 14 monoclonal antibodies (Mabs) that react with surface antigens of *Pediococcus* beer spoilage organisms, including *P. damnosus*, *P. pentosaceus*, *P. acidilactici*, and unidentified isolates. In most cases, the Mabs were shown to bind to *Pediococcus* surface antigens, and it was concluded that they show good potential for rapid, sensitive, and specific immunoassay detection of *Pediococcus* beer spoilage organisms. Whiting et al. (1992) detected diacetyl-producing pediococci in brewery pitching yeast using an immunofluorescent antibody technique.

Chaban et al. (2002) used immunoblotting for identification of beer spoilage pediococci and could also detect *Pediococcus claussenii*. They analyzed 31 *Pediococcus* isolates, most of brewing origin, by immunoblots using polyclonal antisera generated in mice or rabbits against various *Lactobacillus* and *Pediococcus* isolates. The survey immunoblots revealed a unique banding pattern for the isolate representing each phylogenetic group, confirming that isolates identified phylogenetically as *P. acidilactici*, *P. damnosus*, *P. pentosaceus*, and the new *Pediococcus* species, *P. claussenii*, can be readily identified by immunoblotting.

Walter et al. (2001) detected *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis (DGGE) of DNA fragments generated by PCR with 16S ribosomal DNA-targeted group-specific primers. Comparison of PCR-DGGE results with those of bacteriological culture showed that the food-associated species could not be cultured from the fecal samples by plating on Rogosa agar, while all LAB species (including pediococci) cultured from feces were detected in the DGGE profile.

Pediococci may be enumerated and identified simultaneously using DNA probe techniques, e.g., of glucan-forming wine-spoilage strains of *P. damnosus* (Lonvaud-Funel et al., 1993) or of

non-glucan-forming strains of *P. damnosus* and *P. pentosaceus* in fermenting grape must and wine (Lonvaud-Funel et al., 1991). These methods are also successfully used for detection of strains of *P. pentosaceus* in silage (Cocconcelli et al., 1991). Fortina et al. (1997) identified *Pediococcus acidilactici* and *Pediococcus pentosaceus* using 16S rRNA and *ldh*D gene-targeted multiplex PCR analysis.

Identification

Phenotypic Characterization

All true members of the genera *Pediococcus* and *Tetragenococcus* are Gram-positive; cytochromes are absent, and all are catalase-negative, which was also confirmed by showing the absence of cytochromes by the benzidine test. Strains of some species, however, display pseudocatalase activity especially when grown on media with low carbohydrate content (Weiss, 1992). They are facultatively aerobic and produce lactic acid as major end product of glucose fermentation by the Embden-Meyerhof pathway, either to DL-, or in the case of *P. claussenii* and the tetragenococci, to L(+)-lactic acid. Gas is not produced from glucose. The cells are spherical and uniform in size, and are never elongated cocci; they divide alternately in two planes at right angles to form tetrads, which, however, may not always be easily detectable. Quite frequently, only single cells, pairs and clusters of cells can be observed microscopically. Microscopic examination should be preferably by phase-contrast microscopy and of cultures grown in MRS broth (or modifications of MRS, e.g., with added NaCl, etc.) rather than other media commonly used, e.g., those used by clinical laboratories. They are nonmotile, do not form spores or capsules, and do not reduce nitrate. These features may separate the pediococci and tetragenococci from all other Gram-positive cocci.

The key characteristics for distinguishing between the genera *Pediococcus*, *Tetragenococcus* and *Aerococcus* and the atypical species "*P. dextrinicus*" are shown in Table 1. Phenotypically, the genera *Pediococcus* and *Tetragenococcus* are separated mainly on the basis of the halotolerant nature of the tetragenococci and the acidophilic to acidotolerant nature of the pediococci.

A simplified, dichotomous key for the phenotypic differentiation of species within the genera *Pediococcus* and *Tetragenococcus* is shown in Fig. 3. Characteristics generally used for the identification of species of these genera include the range of temperatures (35°, 40°, 45° and 48°C), pH (pH 4.5, 7.0 and 8.0), NaCl concentration at which growth occurs, and physiological characteristics such as fermentation of different carbohydrates, hydrolysis of arginine, and the isomer(s) of lactic acid produced. The key characteristics that have proven most useful for the identification to species level are given in Table 6. However, even strains with high DNA-DNA similarity may reveal a broad variability within their phenotypic properties, and thus, no single characteristic can be taken as absolutely discriminatory (Weiss, 1992). Therefore, the more comprehensive phenotypic information supplied in Table 3 may be vital for decision making on a phenotypic basis.

Numerical analysis of total soluble cell protein patterns on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was originally developed by Kersters and De Ley (1980) and has been used for the identification of numerous bacterial species. This is still one of the major tools in polyphasic taxonomy. Provided this technique is carried out with a set of well-defined reference strains together with constant approach in the numerical analysis, it enables reliable species identification. Using comparison of SDS-PAGE whole-cell protein patterns with a SDS-PAGE database of LAB, Leisner et al. (1999) identified pediococci among 92 strains of different LAB isolated from a Malaysian food ingredient, chili bo.

Beverly et al. (1997) used pyrolysis mass spectrometry for fatty acid analysis of beer spoiling microorganisms. In situ thermal hydrolysis-methylation (THM) of whole bacteria with tetramethylammonium hydroxide (TMAH) produced diagnostic fatty acid methyl ester (FAME) profiles for *Pediococcus damnosus*, *P. dextrinicus* and *Lactobacillus brevis*. It was reported that fatty acids from bacteria in beer could be analyzed without the need for a fatty acid extraction or chromatography procedure, providing FAME profiles for bacteria in beer at a concentration of 10^{-7} colony forming units (CFU)/ml of beer.

Molecular Techniques

Ribotyping (Grimont and Grimont, 1986) may be applied as a useful taxonomic tool for typing of pediococci. Both riboprinting (using a Qualicon RiboPrinter) and 16S rRNA gene sequencing were used to identify brewery *Pediococcus* isolates by Barney et al. (2001). Out of 46 isolates, 41 were identified as *Pediococcus damnosus* using *Eco*RI digestion. These results were compared with those obtained from PCR amplification of the complete 16S rRNA gene, and it was concluded that, although 16S rRNA gene sequencing correctly identified the genus and species of the test *Pediococcus* isolates, riboprinting proved to be a better method for subspecies differentiation.

Gram-positive cocci

Uniformly spherical cells
(tetrad formation / pairs, no chains)

Catalase-neg., ovoid cells
(pairs and chains)

Catalase
hippurate hydrolysis
+   −

CO$_2$ from glucose
−   +

Aerococcus   *Pediococcus / Tetragenococcus*

Streptococcus   *Leuconostoc*
Lactococcus   *Weissella*
Enterococcus

Growth at pH 9; growth tolerance to 10–18% NaCl
−   +

Pediococcus (no CO$_2$ from gluconate*)   *Tetragenococcus*

Fermentation of rhamnose,
and arabinose and/or xylose
+   −

Fermentation of melezitose, sucrose,
maltotriose and xylose; tolerance to >18%
NaCl; growth at 40°C
+   −

Growth at 48°C /
Maltose fermentation

T. halophilus   *T. muriaticus*

+   −
−   +
P. acidilactici   *P. pentosaceus*

Fermentation of galactose / ribose
Lactic acid isomer from glucose
+ / −   − / +
DL   L (+)

Growth at pH 7.0; at 35°C
−   +

P. claussenii

P. damnosus   Fermentation of lactose
−   +

P. parvulus   *P. inopinatus*

*The "atypical" species *P. dextrinicus* is able to decarboxylate gluconate and also to hydrolyze starch.

Fig. 3. Key to the phenotypic differentiation of species within the genera *Pediococcus* and *Tetragenococcus*.

Satokari et al. (2000) used a RiboPrinterR System for ribotyping 18 *Pediococcus* strains, mainly of brewery origin, after they were first identified by phenotypical characterization (API 50 CHL; bioMérieux, Marcy-l'Etoile, France) and SDS-PAGE profiling. Ribotype identifications were confirmed by 16S rDNA sequencing of selected strains. Ribotyping was found to be more discriminative than phenotypical identification methods. Moreover, automated ribotyping was found to be a rapid and reliable method for identifying pediococci, even when construction of a comprehensive fingerprint library was required.

Table 6. Key for the presumptive identification of species of the genera *Pediococcus* and *Tetragencoccus*.

I. a) Growth at pH 9.0 but not at 4.5, growth tolerance to <10% NaCl, ribose fermented	II
I. b) Growth at pH 4.5 but not at 9.0	III
II. a) Growth in 15% NaCl, fermentation of melezitose, sucrose	*T. halophilus*
II. b) No growth in 15% NaCl, no fermentation of melezitose or sucrose	*T. muriaticus*
III. a) Fermentation of rhamnose, and arabinose and/or xylose	IV
III. b) Rhamnose, and arabinose and/or xylose not fermented	V
IV. a) Growth at 48°C, maltose not fermented	*P. acidilactici*
IV. b) No growth at 48°C, maltose fermented	*P. pentosaceus*
V. a) Fermentation of ribose but not of galactose; L(+) lactate from glucose	*P. claussenii*
V. b) Fermentation of galactose but not of ribose, DL-lactate from glucose	VI
VI. a) Growth at 35°C and at pH 7.0	VII
VI. b) No growth at 35°C or at pH 7.0	*P. damnosus*
VII. a) Lactose fermented	*P. inopinatus*
VII. b) Lactose not fermented	*P. parvulus*

Nigatu et al. (1998) used a PCR-based RAPD technique for discrimination of *Pediococcus pentosaceus* and *P. acidilactici*, and for rapid grouping of 116 *Pediococcus* isolates from fermenting tef dough and fermented kocho. Mora et al. (2000) developed a molecular RAPD marker for the identification of *Pediococcus acidilactici* strains, using a single primer targeted to the pediocin AcH/PA-1 gene.

Amplified 16S rDNA restriction analysis (16S-ARDRA) was used as a tool for identification of LAB isolated from grape must and wine by Rodas et al. (2003). The protocol was based on the direct amplification of 16S rDNA from a colony and the later digestion with one of the restriction enzymes *Bfa*I, *Mse*I and *Alu*I. A sequential use of the three enzymes is proposed to simplify identification of LAB in wine: first *Mse*I, then *Bfa*I and finally, if necessary, *Alu*I. The technique enabled the identification of *Pediococcus parvulus* and *P. pentosaceus* among 342 LAB isolates (also comprising strains of *Lactobacillus brevis*, *L. collinoides*, *L. coryniformis*, *L. bilgardii*, *L. mali*, *L. paracasei*, *Leuconostoc mesenteroides* and *Oenococcus oeni*) from musts and wines.

Miambi et al. (2003) used an integrated approach of culture-dependent and culture-independent methods (e.g., DGGE) for the identification, isolation and quantification of representative bacteria including *Pediococcus* spp. from fermented cassava dough.

Ennahar et al. (2003) studied the phylogenetic diversity of LAB associated with paddy rice silage, using 16S ribosomal DNA analysis. The 161 LAB isolates from whole-crop paddy rice silage were subjected to phenotypic and genetic analyses, and the virtually complete 16S rRNA gene was PCR amplified and sequenced. Among the LAB, 11% could be identified as *Pediococcus acidilactici* and 2% as *Pediococcus pentosaceus*. Also Chenoll et al. (2003) applied rDNA-based techniques for identification of *Carnobacterium*, *Lactobacillus*, *Leuconostoc* and *Pediococcus*. These techniques included the analysis of profiles generated by intergenic spacer region (ISR) amplification, ISR restriction, and ARDRA and have been evaluated as molecular tools for identifying the mentioned genera. This technique could not be used to distinguish between the genera *Lactobacillus* and *Pediococcus*. The *Pediococcus* species were differentiated using ISR-*Dde*I profiles.

Ogier et al. (2002) identified the bacterial population in dairy products by temporal temperature gradient gel electrophoresis (TGGE). The strains studied included members of the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Pediococcus*, *Streptococcus* and *Staphylococcus*. TTGE was shown to distinguish bacterial species in vitro, as well as in both liquid and solid dairy products.

Simpson et al. (2002) studied the genomic diversity of 33 previously assigned strains from six species within the genus *Pediococcus*, as revealed by RAPD PCR and PFGE. The RAPD PCR patterns produced by two separate random primers, termed "P1" (ACGCGCCCT) and "P2" (ATGTAACGCC), were compared by the Pearson correlation coefficient and the unweighted pair group method with arithmetic averages clustering algorithm. Assigning each strain to a type strain with which it shared the highest level of similarity, both primers grouped 17 of the 27 strains to their proposed species. Following genomic digestion with the restriction enzymes *Apa*I, *Not*I and *Asc*I, PFGE produced 30, 32 and 28 distinct macrorestriction patterns, respectively.

Maintenance and Conservation of Cultures

The general procedures of cultivation, maintenance, and preservation as used for lactobacilli, leuconostocs and weissellas (see The Genera *Lactobacillus* and *Carnobacterium* along with

The Genera *Leuconostoc*, *Oenococcus* and *Weissella* both in this Volume) are also applicable to most pediococci.

MRS broth or agar is generally used for axenic cultivation of pediococci, and cultures may be stored as stab cultures (MRS-agar) at 4°C with transfers every 1–2 weeks. Vitality may be retained in yeast glucose litmus milk + calcium carbonate for several months (Sharpe, 1981). Juven (1979) recommended the preservation of stock cultures, partly dehydrated, on granular pumice stone for 6–24 months at room temperature. Medium-term conservation of at least one year may be obtained by mixing a well-developed but not overgrown culture with an equal volume of sterile glycerol in a small screw-capped vial, followed by storage at –20°C. Loops of inoculum can thus be taken without exposing the cells to the killing effect of the freezing or thawing procedure (Weiss, 1992). Initial freezing at –70°C, followed by the addition of protecting agents such as sterile milk or cream (18%) may also give good results; Dobson et al. (2002) recommended storage of cultures at –70°C in double-strength skim milk. Experience over several years has also proved the value of cryopreservation at –80°C for practically all LAB. A freshly grown (18–24 h) cell suspension is washed twice in a phosphate buffer (e.g., quarter-strength Ringer's solution), resuspended in the fresh growth medium containing 10–15% sterile glycerol, and distributed in small cryotubes before freezing. For long-term conservation, lyophilization by standard procedures or preferably storage in liquid nitrogen is advisable.

For conservation of *Tetragenococcus* strains, Kobayashi et al. (2000) recommend stabbing the inoculum into 13% (w/v) NaCl-GYP agar medium, and storage at 5°C with subculturing every 6 months. Strains of *T. muriaticus* were maintained as stab cultures in 10% NaCl-GYP agar supplemented with 10% NaCl, 1% MgSO$_4$, 0.1% KCl, 0.5% CaCO$_3$ and 0.3% agar. The pH was adjusted to 7.0 with 1N NaOH. Strains were stored at 4°C and transferred to fresh medium every third month.

More information on general cultivation and maintenance of most *Pediococcus* strains is provided by Schillinger and Holzapfel (2003).

Physiology

Pediococci are micro-aerophilic and ferment glucose under anaerobic conditions to DL or L(+)-lactic acid. *Pediococcus dextrinicus* (Simpson and Taguchi, 1995) and *P. claussenii* are the only recognized *Pediococcus* species that produce only L(+)-lactate from glucose. *Pediococcus urinae-equi* (which is more closely related to *Aerococcus viridans* than to other *Pediococcus* spp.) also produces only L(+)-lactic acid. All species grow in the presence of 5% (w/v) NaCl, with *P. acidilactici*, *P. pentosaceus* and "*P. urinae-equi*" being able to withstand 10% NaCl (Simpson and Taguchi, 1995). Sensitivity to NaCl varies with composition of growth medium and incubation conditions (Nakagawa and Kitahara, 1959; Coster and White, 1964). Of all species, only "*P. urinae-equi*" does not grow at pH 4.5. *Pediococcus* species (except *P. damnosus*) grow at pH 7.5 (Simpson and Taguchi, 1995). Only some strains of *P. acidilactici* and *P. pentosaceus*, but all strains of "*P. urinae-equi*," grow at pH 8.5. Tolerance to ethanol is characteristic to certain strains of *P. damnosus*, *P. inopinatus* and *P. parvulus* (Davis et al., 1988). Pediococci are catalase negative, although some strains of *P. pentosaceus* and "*P. urinae-equi*" produce catalase or pseudocatalase (Simpson and Taguchi, 1995). Nitrate is not reduced and indole is not formed from tryptophan (Nakagawa and Kitahara, 1959). Hippurate is not hydrolyzed, except by "*P. urinae-equi*" (Tanasupawat and Daengsubha, 1983). Arginine hydrolysis is rare and has only been recorded for *P. acidilactici* and *P. pentosaceus* (Simpson and Taguchi, 1995).

Carbohydrate fermentation is homofermentative, i.e., no CO$_2$ production from glucose (Simpson and Taguchi, 1995). Only one species, *P. dextrinicus*, produces CO$_2$ from gluconate. Fructose, mannose and cellobiose are fermented by all species. Although most species ferment galactose and maltose, a few strains of *P. damnosus*, *P. parvulus* and *P. claussenii* are unable to do so (Simpson and Taguchi, 1995; Dobson et al., 2002). Rhamnose, melibiose, melezitose, raffinose, inulin, and α-methyl glucoside-D are not readily fermented. Sucrose is fermented by all species, except *P. inopinatus*, *P. parvulus*, *P. pentosaceus* and *P. claussenii* (Simpson and Taguchi, 1995; Dobson et al., 2002). Starch is only fermented by *P. dextrinicus*, most probably because of the activity of α-amylase, glucoamylase or other extracellular enzymes. Only three species, *P. acidilactici*, "*P. urinae-equi*" and *P. claussenii* ferment mannitol (Simpson and Taguchi, 1995; Dobson et al., 2002). Sorbitol is only fermented by some strains of "*P. urinae-equi*." Variable results were recorded for the fermentation of trehalose, lactose, maltotriose, dextrin, glycerol, salicin and amygdalin (Simpson and Taguchi, 1995). In the presence of oxygen, *P. pentosaceus* ferments glycerol to lactate, acetate, acetoin and CO$_2$ (Dobrogosz and Stone, 1962). The pentoses (arabinose, ribose and xylose) are not fermented by *P. damnosus*, *P. dextrinicus*, *P. inopinatus* and *P. parvulus*. Acetoin production varies and is strain specific. *Pediococcus parvulus* and "*P. urinae-equi*" do not produce acetoin. Exopolysaccharide production from carbohydrates has been

reported for strains of *P. pentosaceus* isolated from Argentinian wine (Manca de Nadra and Strasser de Saad, 1995) and Thai fermented pork sausage (Smitinont et al., 1999).

Carbohydrate fermentation has been the best studied for *P. pentosaceus*. Glucose is transported across the cell membrane by using the phosphoenolpyruvate (PEP)-phosphotransferase (PTS) system and metabolized via the Embden-Meyerhof-Parnas pathway to yield D(−) and L(+)-lactate as the main end product (Romano et al., 1979). In the presence of L-malate, additional L(+)-lactate is produced (Radler et al., 1970). In the presence of pentoses, *P. pentosaceus* produces equal concentrations of lactate and acetate (Fukui et al., 1957). The production of diacetyl has been recorded for *P. damnosus*, and under growth-limiting conditions, acetoin may also be produced. Acetate is rarely produced and has only been recorded for certain strains of *P. pentosaceus* grown in the presence of oxygen (Thomas et al., 1985).

Although pediococci prefer micro-aerophilic to anaerobic growth, they are aerotolerant and may be classified as "aerotolerant anaerobes." Catalase is normally not produced and superoxide dismutase is not formed. However, a pseudocatalase has been noted for some strains of *P. pentosaceus* (Whittenbury, 1964). More than half the number of *P. pentosaceus* strains isolated from goat milk, Feta and Kaseri cheese had a weak catalase activity (Tzanetakis and Litopolou-Tzanetaki, 1989). True catalase activity has been recorded for *P. acidilactici* but only when grown in heme-containing medium (Whittenbury, 1964). Protection against oxygen is achieved when strains are cultured in the presence of high concentrations of Mn(II) (Archibald, 1986).

Pediococci, like most LAB, require complex growth factors and grow best in rich media. The amino acid requirements, as with many other properties of the pediococci, are variable between strains of any specific species (Jensen and Seeley, 1954; Sakaguchi, 1960). Most strains require alanine, aspartic acid, glutamic acid, arginine, histidine, isoleucine, phenylalanine, proline, threonine, tyrosine, valine, tryptophan, cystine, glycine and leucine. Some strains require lysine, methionine and serine. Although some strains produce aminopeptidases, most will only grow in medium supplemented with complex nitrogen sources (Tzanetakis and Litopolou-Tzanetaki, 1989). Certain strains of *P. pentosaceus* produce proteases, dipeptidases, dipeptidyl aminopeptidases and aminopeptidases but not carboxypeptidases or endopeptidases (Bhowmik and Marth, 1990). Casein hydrolysis among these strains is specific, as recorded by the partial hydrolysis of α_{SI}-casein and complete hydrolysis

of β-casein (Bhowmik and Marth, 1990). *Pediococcus parvulus*, on the other hand, does not possess proteolytic activity (Davis et al., 1988). Strains of *P. damnosus* isolated from beer have extracellular peptidases for hydrolysis of polypeptides. Some strains of *P. damnosus* have a unique requirement for mevalonic acid and only grow in the presence of CO_2 (Kitahara and Nakagawa, 1958; Kitahara and Nakagawa, 1959).

All species require nicotinic acid, panthothenic acid and biotin but not thiamine, ρ-aminobenzoic acid, cobalamin, riboflavin, pyridoxine and folinic acid (Sakaguchi and Mori, 1969; Simpson and Taguchi, 1995). The growth of *P. damnosus* is stimulated by pyridoxine (Solberg and Clausen, 1973). Growth is not stimulated by adenine, guanine, uracil and xanthine (Sakaguchi and Mori, 1969). Some strains of *P. pentosaceus* require 5-formyl-tetrahydrofolic acid (5-formyl-THF) as growth factor (Günther and White, 1961b).

As for most LAB, growth is not stimulated by iron (Archibald, 1986). Of the six minerals tested, manganese (Mn^{+2}) is required by all strains, followed by Ca^{+2}, Fe^{+2} and Zn^{+2}. The requirement for Fe^{+3} and Mg^{+2} is about the same as for Zn^{+2} (Raccach, 1981).

Tetragenococcus halophilus is the predominating halophilic LAB species in high-salt fermented foods, followed by *T. muriaticus*, and it has been shown that the high saline concentrations and low pH of these food substrates have significant impact on the growth, lactic acid production, and pH reduction ability of these species (Kobayashi et al., 2004). The interactive support of these two species has been observed in reducing pH in hypersaline or low pH environment. The major physiological differences between the two species were found in the ability of *T. halophilus* to grow in medium not supplemented with NaCl, and in the fermentation of L-arabinose, sucrose (by *T. halophilus*), and D-mannitol (mainly by *T. muriaticus*; Kobayashi et al., 2000).

In a comparative study of physiological responses of LAB subjected to osmotic stress, Baliarda et al. (2003) found that glycine betaine, dimethylsulfonioacetate, choline, proline and L-carnitine relieved growth inhibition at 0.8M NaCl, by efficiently competing with glycine betaine transport. *Tetragenococcus halotolerans* exhibited a larger spectrum of compatible solutes than non- or less salt tolerant LAB, and Baliarda et al. (2003) concluded that restoration of growth by ectoine under osmotic constraint appears to be specific to the genus *Tetragenococcus*.

Physiological diversity among strains of *T. halophilus* was reported by Gürtler et al. (1998). Among the strains studied, all tolerated 26% NaCl and reached optimum growth rate at pH

7.5. On the other hand, strains responded differently to anaerobic conditions. When hematin was added to aerobic cultures, both the lag phase and generation time were reduced, and the growth yield increased twofold. Heme-dependent catalase activity was detected for 12 out of 21 strains. Moreover, growth conditions strongly affected the composition of the metabolic end products, with anaerobically grown cultures producing lactate, acetate and formate, in contrast to aerobically grown ones that produce mainly acetate. Phosphohydrolase activity seems common to *T. halophilus*. On the other hand, neither proteolytic activity nor certain enzymes (alkaline phosphatase, α- and ß-galactosidases, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase) have been detected (Gürtler et al., 1998).

Biogenic amines (BA) are commonly produced during fermentation as a result of amino acid decarboxylase activities of some strains of LAB. This property is not typical of *T. halophilus*, and Gürtler et al. (1998) reported that none out of 21 strains showed any ability to produce BA in the presence of the respective precursor amino acids. On the other hand, histamine production at NaCl concentrations >10% has been found to be a typical characteristic of *T. muriaticus* (Kimura et al., 2001), after its first isolation and identification by Satomi et al. (1997) as a novel species of histamine-forming halophilic LAB. Its ability to form histamine at low acidity around pH 5.8, and under O_2-limiting conditions, optimal NaCl concentration (5–7%), and glucose >1% has been reported by Kimura et al. (2001). This histidine decarboxylase (each molecule consisting of one 28.8- and one 13.4-kDa polypeptide) was purified and sequenced by Konagaya et al. (2002). Konagaya et al. (2002) found the N-terminal amino acid sequences of these polypeptides were highly correlated with those of the α- and ß-chains of other Gram-positive bacterial histidine decarboxylases.

Genetics

Since the first description of plasmids for the genus *Pediococcus* (Graham and McKay, 1985), many genes encoding the fermentation of sugars have been characterized. In the case of *P. pentosaceus*, the genes encoding the fermentation of raffinose, melibiose, sucrose and often lactose are located on plasmids (Gonzalez and Kunka, 1986; Hoover et al., 1988). For some strains of *P. acidilactici*, the fermentation of sucrose is also encoded by genes located on plasmids (Hoover et al., 1988). The gene encoding erythromycin resistance was detected on a 40-MDa plasmid in *P. acidilactici* (Torriani et al., 1987). Frequent transfer of plasmids between *Pediococcus* and *Enterococcus*, *Streptococcus* and *Lactococcus*

spp. have been observed (Gonzalez and Kunka, 1983), and plasmid transfer may very well be a natural process of gene exchange among these genera. The first electroporation of plasmids into *Pediococcus* was reported by Kim et al. (1992).

Information on bacteriophages of pediococci (unlike other genera of LAB) is limited to reports of phages for *P. halophilus* (Uchida and Kanbe, 1993) and *P. acidilactici* (Caldwell et al., 1999). The bacteriophages of *P. acidilactici* (Caldwell et al., 1999) were temperate but could be induced with mitomycin C. The phages were classified into two genetic groups (Caldwell et al., 1999). Most of the genetic studies conducted on *Pediococcus* spp. revolves around the genes encoding different bacteriocins.

The majority of bacteriocins (pediocins) produced by pediococci are grouped into Class IIa, i.e., small (less than 10 kDa), non-lanthionine-containing and *Listeria*-active peptides with an YGNGV-consensus sequence (tyrosine-glycine-asparagine-glycine-valine) in the N-terminus (Nes et al., 1996). Pediocin PD-1, first described by Green et al. (1997) and later partly sequenced by Bauer (2004), may be a lantibiotic and thus a Class I bacteriocin. Pediocins are membrane-active and resistant to temperatures of up to 100°C. A few pediocins withstand autoclaving, i.e., 15 min at 121°C. Pediocin precursor molecules are characterized by a double glycine (G-G) processing site (Klaenhammer, 1993) and have a high content of small amino acids which renders the peptide strongly cationic (its pI is between 8 and 11; Abee, 1995a; Miller et al., 1998). Hydrophobic and amphiphilic domains are clearly distinguished (Abee, 1995a).

Pediocin PA-1 is the best characterized of all pediocins and all other bacteriocins within Class IIa (Gonzalez and Kunka, 1987; Rodriques et al., 2002). Pediocins share many sequence similarities with bacteriocins produced by *Lactobacillus* spp., e.g., curvacin A, sakacin P, bavaricin A, and bavaricin MN; *Leuconostoc* spp., e.g., leucocin A and mesentericin Y105; *Streptococcus* spp., e.g., mundticin; *Enterococcus* spp., e.g., enterocin A; *Weissella* spp., e.g., piscicolin 126; and *Carnobacterium* spp., e.g., carnobacteriocin B2 (Table 7).

Pediocin AcH, produced by *P. acidilactici* LB42-923 isolated from fermented sausage (Bhunia et al., 1988), is identical to pediocin PA-1 (Marugg et al., 1992; Bukhtiyarova et al., 1994). Pediocin PA-1 production has also been recorded for strains of *P. parvulus* isolated from minimally processed vegetables (Bennik et al., 1997), and a strain of *P. acidilactici* isolated from commercial starter cultures (Nieto Lozano et al., 1992). Pediocin N5p, produced by *P. pentosaceus* isolated from wine, differs from pediocins PA-1 (and AcH) by adsorbing to Gram-positive and Gram-negative cells (Manca de Nadra et al.,

Table 7. Amino acid sequences of Class IIa bacteriocins (containing the YGNGV consensus sequence) with anti-Listeria activity.

Bacteriocin	Leader peptide[a]	Mature peptide	References
Acidocin A	MISMISSHQKTLTDKELALISGG	KTYYGTNGVHCTKTKKSLWGKVRLKNVIPGTLCRKQSLPIKQDLKILLG WATGAFGKTFH	Kanatani et al., 1995a
Bavaricin A	na	KYYGNGVHXGKHSXTVDWGTAIGNIGNNAAANXATGXNAGG	Larsen et al., 1993
Bavaricin MN	na	TKYYGNGVYXNSKKXWVDWGQAAGGIGQTVVXGWLGGAIPGK	Kaser and Montville, 1996
Bifidocin B	na	KYYGNGVTCGLHDCRVDRGKATCGIINNGGMWGDIG	Yildirim et al., 1999
Carnobacteriocin B2[b]	MNSVKELNVKEMKQLHGG	VNYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP	Quadri et al., 1994
Carnobacteriocin BM1[c]	MKSVKELNKKEMQQINGG	AISYGNGVYCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH	Quadri et al., 1994
Curvacin A[d]	MNNVKELSMTELQTITGG	ARSYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	Tichaczek et al., 1993
Divercin V41	MKNLKEGSYTAVNTDELKSINGG	TKYYGNGVYCNSKKCWVDWGQASGCIGQTVVGGWLGGAIPGKC	Métivier et al., 1998
Enterocin A	MKHLKILSIKETQLIYGG	TTHSGKYYGNGVYCTKNKCTVDWAKATTCIAGMSIGGFLGGAIPGKC	Aymerich et al., 1996
Leucocin A[e]	MNNMKPTESYEQLDNSALEQVVGG	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNFW	Hastings et al., 1991
Leucocin Ta11a	MNNMKSADNYQQLDNNALEQVVGG	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW	Felix et al., 1994
Mesentericin Y 105[f]	MTNMKSVEAYQQLDNQNLKKVVGG	KYYGNGVHCTKSGCSVNWGEAASAGIHRLANGGNGFW	Fleury et al., 1996
Mundticin	na	KYYGNGVSCNKKGGCSVDWGKAIGIIGNNSAANLATGGAAGWSK	Bennik et al., 1998
Pediocin PA-1[g]	MKKIEKLTEKEMANIIGG	KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC	Marugg et al., 1992
Piscicocin V1a[h]	na	KYYGNGVSCNKNGCTVDWSKAIGIIGNNAAANLTTGGAAGWNKG	Bhugaloo-Vial et al., 1996
Plantaricin C19	na	KYYGNGLSCSKKGCTVNWGQAFSCGVNRVATAGHGK	Atrih et al., 1993
Sakacin P[i]	MEKFIELSLKEVTAITGG	KYYGNGVHCGKHSCTVDWGTAIGNIGNNAAANWATGGNAGWNK	Tichaczek et al., 1994

Abbreviations: na, not available; A, alanine; R, arginine; N, asparagine; D, aspartate; C, cysteine; Q, glutamine; E, glutamate; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; and V, valine.

[a]Leader peptide where available.
[b]Carnobacteriocin B2 = Carnocin CP52 (Herbin et al., 1997).
[c]Carnobacteriocin BM1 = Piscicolin V1b (Bhugaloo-Vial et al., 1996) = Carnocin CP51 (Herbin et al., 1997).
[d]Curvacin A = Sakacin A (Holck et al., 1992).
[e]Leucocin A = Leucocin A-TA33a (Papathanasopoulos et al., 1998).
[f]Mesentericin Y105 = Mesentericin 52A (Revol-Junelles et al., 1996).
[g]Pediocin PA-1 = Pediocin AcH (Motlagh et al., 1992).
[h]Piscicocin V1a = Piscicolin 126 (Jack et al., 1996).
[i]Sakacin P = Sakacin 674 (Holck et al., 1994b).

Fig. 4. Schematic representation of the pediocin PA-1 operon. The promoter (➤) and filled arrows indicate the individual genes and the direction of transcription. From Marugg et al. (1992).

1998). Pediocin ST18, produced by *P. pentosaceus* (Todorov and Dicks, 2004) could be related to pediocin PA-1. Pediocin AcM, produced by *P. acidilactici*, may be different from pediocin PA-1, although preliminary data suggests some degree of similarity on the DNA level (Elegado et al., 1997). Bacteriocin ACCEL, isolated from *P. pentosaceus* (Wu et al., 2004), may also be related to pediocin PA-1. The only pediocin really different from all other pediocins thus far described is pediocin A, produced by *P. pentosaceus* FBB61 (=ATCC 43200). Compared to the other pediocins which are in the size range of approx. 4 kDa (Ray, 1994), pediocin A with a molecular mass of 80 kDa (Giacomini et al., 2000) groups with the larger bacteriocins of group III (Nes et al., 1996). Pediocin A is encoded by a 19,515-bp plasmid, pMD136 (Giacomini et al., 2000).

Sequencing of the pediocin PA-1 operon revealed four open reading frames (ORFs), designated "*pedA*," "*pedB*," "*pedC*" and "*pedD*," with an upstream promoter area (Fig. 4). The structural gene, *pedA*, encodes pre-pediocin PA-1, the precursor of pediocin and its leader peptide, a protein of 62 amino acids. *pedB* encodes the immunity protein, consisting of 112 amino acids. *pedC* encodes an accessory protein of 174 amino acids involved in protein externalization, while *pedD* encodes an ATP-binding cassette (ABC) transporter protein of 724 amino acids (Marugg et al., 1992).

All Class II bacteriocins are produced as precursors with an N-terminal extension (Van Belkum and Stiles, 1995). The leader sequences of some bacteriocins are identical, e.g., the two pediocins (Marugg et al., 1992; Motlagh et al., 1994) and sakacin A and curvacin A pre-peptides (Tichaczek et al., 1992; Tichaczek et al., 1993; Table 7). The extremely high homology between the various leader sequences suggests that for similar types of bacteriocins from the same species, the amino acid sequences of the leader peptides may be particularly highly conserved. Apparently, there has been a remarkably consistent conservation of both the hydrobicity and charge characteristics of the individual bacteriocins.

The immunity gene encodes a protein that protects the producer organism from its own mature bacteriocin (Nes et al., 1996). Potential immunity proteins have been identified next to or downstream from all bacteriocin structural genes studied. Immunity genes not directly associated with the bacteriocin cluster have also been identified (Eijsink et al., 1998). Variation in the presence and expression of these genes may account for the large variation in sensitivity displayed by LAB towards bacteriocins. Immunity proteins range in size from 51 to 150 amino acids. While significant homology exists among the structural genes of the pediocin-like bacteriocins, this trend does not occur with immunity genes, although some resemblances do occur (Aymerich et al., 1996; Moll et al., 1999; Van Reenen et al., 2002). The mechanism of action of immunity proteins is not currently understood but could entail the shielding of a receptor, prevention of pore formation, pore blocking, or bacteriocin degradation (Moll et al., 1999).

Bacteriocins containing the double-glycine type leader sequences (G-G) are translocated by a dedicated export system identified as ABC transporters (Fath and Kolter, 1993; Nes et al., 1996). The gene encoding the bacteriocin ABC transporter is usually part of the bacteriocin operon or can be found on an operon near the bacteriocin operon (Nes et al., 1996). ABC transporters facilitate the secretion of a wide range of products in both prokaryotic and eukaryotic organisms. These products include periplasmic permeases (bacterial importers), which transport oligopeptides, amino acids, sugars, phosphate, metal ions, and vitamins; eukaryotic exporters, which transport lipophilic drugs, peptides and pigments; and bacterial exporters, which transport molecules such as large protein toxins, small peptide antibiotics, polysaccharides, antibiotics, and possibly heme molecules (Fath and Kolter, 1993).

The bacteriocin ABC transporters have a dual function, facilitating both the removal of the leader peptide from its substrate and the transport of the substrate across the cytoplasmic membrane (Håvarstein et al., 1995). Bacteriocin ABC-transporters contain three domains on the same polypeptide, consisting of a cytoplasmic N-terminal proteolytic domain, a hydrophobic integral membrane domain, and a cytoplasmic C-terminal ATP-binding domain (Håvarstein et al., 1995; Nes et al., 1996; Fig. 5). Two polypeptides appear to be required for the bacteriocin ABC transporter to be functional (Håvarstein et al., 1995).

The membrane-spanning domains (MSDs) consist of six membrane-spanning segments (Fig. 5). ABC transporters (or traffic ATPases) use ATP hydrolysis as a source of energy required for translocation and have a highly conserved ATP-binding cassette (Fath and Kolter, 1993).
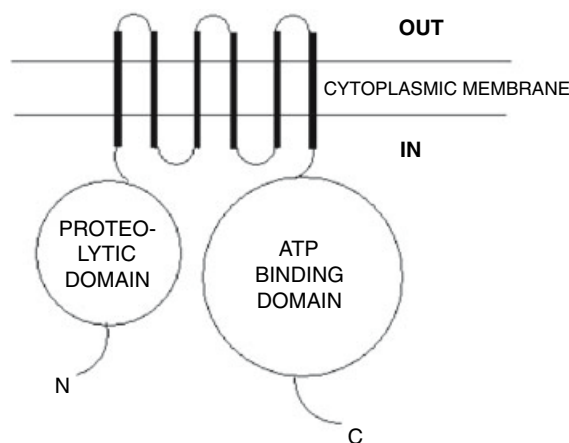
Fig. 5. ATP binding cassette translocator with the N-terminal proteolytic domain, six membrane-spanning domains and the ATP-binding domain in the C-terminus of a single polypeptide. (Redrawn from Håarstein et al., 1995.)

The conserved ATP-binding motif can be found in all the ABC transporters of the bacterial export subfamily. Removal of the leader peptide from its substrate and the subsequent translocation of the bacteriocin across the cytoplasmic membrane effectively prevent the mature and active bacteriocins from remaining in the cytoplasm (Håarstein et al., 1995).

It is proposed that bacteriocin-mediated transmembrane ion flow results in cytotoxic effects, causing a drop in the intracellular pH and inhibiting enzymatic processes. An influx of cytotoxic sodium ions and ATP depletion due to futile cycles are caused by ion gradient dissipation. Dissipation of the proton motive force and the transmembrane potential arrest processes dependent on these gradients (Bruno and Montville, 1993; Moll et al., 1999).

Bacteriocins often form pores in the membranes of target cells (Abee, 1995a; Abee et al., 1995b). It is hypothesized that the mode of action involves various steps such as binding, insertion and pore formation (Montville and Chen, 1998). Binding of the bacteriocin to the target membrane is necessary for subsequent insertion and pore formation. Although the interaction of a receptor-like factor has been implicated for pediocin PA-1 (Chikindas et al., 1993), a protein receptor does not appear to be essential for binding. Studies by Breukink et al. (1999) have indicated that nisin specifically interacts with the membrane-anchored cell wall precursor Lipid II. Chen et al. (1997) suggested that the binding step primarily involved electrostatic interactions between positive areas of amino acid groups in the bacteriocin and negatively charged phospholipid groups in the target mem-

brane. Jack et al. (1995) also implied that anionic cell surface molecules in the cell wall of Gram-positive bacteria might play a role in the initial interaction with cationic bacteriocins. Analysis of chimers that consist of pediocin-like peptides indicated that the C-terminal part of the molecule is responsible for target specificity (Fimland et al., 1996). A C-terminal fragment of pediocin PA-1 inhibited the activity of pediocin PA-1 peptide, indicating that this fragment competed with the intact peptide for binding sites on the target membrane (Fimland et al., 1998).

Specific amino acids play a role in the antimicrobial activity of Class IIa bacteriocins. The presence of cysteines in the structure of these bacteriocins with subsequent modification of pairs of cysteine residues to form disulfide bridges affects the activity of bacteriocins (Miller et al., 1998). Comparative studies by Eijsink et al. (1998) showed that pediocin PA-1 and enterocin A, which both contain two disulfide bonds, were more active than sakacin P and curvacin A, which contain only one disulfide bond. Pediocin PA-1 lost its activity completely when its sulfide bonds were reduced with dithiothreitol (Chikindas et al., 1993).

Aromatic amino acids are also involved with antimicrobial activity. Removal of tryptophan from the C-terminus of mesentericin Y105 (Fleury et al., 1996), substitution of phenylalanine with serine in carnobacteriocin B2 (Quadri et al., 1997), and substitution of tryptophan with arginine in pediocin PA-1 (Miller et al., 1998) resulted in reduction of activity of these bacteriocins. Loss of activity when small fragments of the N-terminal or the C-terminal are removed suggests that the whole sequence of the bacteriocin is necessary for activity (Fleury et al., 1996; Miller et al., 1998).

Two models are proposed for pore-formation by the Class II bacteriocins. It is thought that the bacteriocins may form a barrel-stave-like bundle of α-helical peptides upon membrane insertion, causing the formation of a pore (Moll et al., 1999). The presence of a helix-breaking amino acid residue in the middle of their sequence may facilitate the insertion of the peptide into the membrane from an initial surface bound state. The hydrophilic faces of a bundle of amphipathic α-helical peptides form the inner wall of the water-filled pore. The outer hydrophobic side of the helical bundles is oriented towards the fatty acyl chains of the membrane lipids (Moll et al., 1999).

Alternatively, a carpet-like model could explain membrane pore formation. Single peptide molecules might be oriented parallel to the membrane surface and interfere with the membrane bilayer organization without forming a peptide aggregate. Once sufficient peptides are

present, temporary membrane collapse due to a strong phospholipid mobilizing activity occurs, resulting in a local and transient permeability (Moll et al., 1999). Homblé et al. (1998) suggested that the negative charge of the membrane lipids confers cation selectivity to such pores.

The pediocin PA-1 operon (from *P. acidilactici* PAC1.0) was expressed in the dairy lactic acid bacterium, *Lactococcus lactis*, to circumvent the need to purify pediocin for application in the dairy industry. Results showed that active pediocin could be produced and secreted by *L. lactis* at a reasonable level when the operon was placed under the control of a lactococcal promoter. The expression of pediocin by *L. lactis* should also enable the production of this bacteriocin during milk fermentation processes. The lactococcin A operon from *L. lactis* has also been expressed in the wild-type, pediocin PA-1-producing *P. acidilactici* PAC1.0. The resulting *Pediococcus* strain produced lactococcin A in addition to pediocin and thus had a wider spectrum of inhibition and greater application to food fermentation (Chikindas et al., 1995).

Sequencing of plasmid pPLA4 harboring the genes encoding plantaricin 423 produced by *L. plantarum* revealed a four-ORF operon structure similar to pediocin PA-1/AcH of *P. acidilactici* and coagulin from *Bacillus coagulans* (Van Reenen et al., 2002). The first ORF, *plaA*, encodes a 56-amino acid pre-peptide consisting of a 37-amino acid mature molecule, with a 19-amino acid N-terminal leader peptide. The second ORF, *plaB*, encodes a putative immunity protein with protein sequence similarities to several bacteriocin immunity proteins. The *plaC* and *plaD* genes are virtually identical to *pedC* and *pedD* of the pediocin PA-1 operon, as well as *coaC* and *coaD* of the coagulin operon. The high similarity (98–99%) of the plantaricin 423 operon to the pediocin PA-1 and coagulin operons is not that unusual. Leucocin A (Van Belkum and Stiles, 1995) and mesentericin Y105 (Fremaux et al., 1995) are different class IIa bacteriocins, but the organization of the genetic determinants of these bacteriocins is identical. It is tempting to speculate that the coagulin and plantaricin 423 operons arose through horizontal gene transfer of the pediocin operon from *P. acidilactici* to *B. coagulans* I₄ and *L. plantarum* 423, respectively. Furthermore, the 485-mer stretch from nucleotides 186 to 670 in the plantaricin 423 operon could have originated from a recombination event with a native or foreign operon encoding a plantaricin C19 or leucocin A-like bacteriocin and its immunity protein. The C-terminal 21 amino acids of plantaricin 423 and its immunity protein vary considerably from that of pediocin PA-1/AcH and coagulin. However, the truncated coding region (nucleotides 670–848 in

the plantaricin 423 operon coding for the C-terminal 58 amino acids of the immunity proteins of pediocin and coagulin) is still present in the plantaricin 423 operon but presumably not encoding a functional immunity protein. These features support the notion of a recombination event subsequent to the horizontal transfer of the pediocin operon to *L. plantarum* 423. These results also support the findings of Le Marrec et al. (2000) that intergeneric transfer of genetic material might have occurred. Considering *B. coagulans* has been isolated from bovine rumen (Le Marrec et al., 2000) and that *P. acidilactici* and *L. plantarum* 423 are likely to have been rumen residents at some point during their evolution, the rumen most likely acted as an ideal niche where high microbial population densities were sufficient for frequent horizontal transfer events to occur (Garcia-Vallve et al., 2000).

Plantaricin 423 was cloned on a shuttle vector under the control of a yeast promoter and heterologously produced in *Saccharomyces cerevisiae* (Van Reenen et al., 2002). The same group also expressed pediocin PA-1 in *S. cerevisiae* (Schoeman et al., 1999).

Epidemiology and Disease

To date, there have been no reports of human disease due to either of the two *Tetragenococcus* species, i.e., *T. halophilus* or *T. muriaticus*. However, *T. muriaticus* isolated from fermented squid liver sauce is known to produce histamine (Satomi et al., 1997; Konagaya et al., 2002). This is a concern from a food safety point of view, as food-borne intoxication with biogenic amines is most frequently caused by histamine (Konagaya et al., 2002; see also under section Physiology).

Since the first documented infections in the 1980s and 1990s, pediococci have been known to sporadically cause infections in humans (Colman, and Efstratiou; 1987; Golledge et al., 1990; Mastro et al., 1990; Riebel and Washington, 1990; Green et al., 1991; Sarma and Mohanty, 1998; Barros et al., 2001; Barton et al., 2001). Although generally considered as harmless, pediococci are typical opportunistic pathogens and can cause infections in persons debilitated as a result of trauma or underlying disease (Mastro et al., 1990; Sarma and Mohanty, 1998). Identification of pediococci as causative agents in disease has also been hampered in the past as a result of the fact that these bacteria were often misidentified as *Streptococcus* species (Ruoff et al., 1988; Golledge et al., 1990; Riebel and Washington, 1990; Barros et al., 2001). This misidentification can be ascribed to difficulties in separating pediococci from physiologically similar bacteria like streptococci, leuconostocs or enterococci (Colman et al., 1987; Ruoff et al.,

1988; Striebel and Washington, 1990; Facklam and Elliot, 1995; Barros et al., 2001). In addition, all these bacteria are intrinsically resistant to vancomycin, which complicates identification but at the same time also increases the need for correct clinical identification (Tankovic et al., 1993; Facklam and Elliot, 1995; Barros et al., 2001).

Pediococcus spp. primarily cause bacteremia and septicemia but in rare cases have also been described as the causative agents of hepatic abscess, pneumonia and possibly meningitis (Mastro et al., 1989; Golledge et al., 1990; Sire et al., 1992; Facklam and Elliot, 1995; Sarma and Mohanty, 1998; Von Witzingerode, 2000; Barton et al., 2001). In one report, a *Pediococcus* sp. was described as a causative agent of septic and gouty arthritis (Yu et al., 2003). Risk factors for *Pediococcus* infection appear to be underlying malignancy or underlying conditions (such as diabetes, pulmonary or vascular disease, hyperparathyroidism, burns or trauma, pregnancy, previous antibiotic treatment, abdominal surgery, tube feeding, pediatric liver transplant, infant jejunoileal atresia, or infant gastroschisis [Mastro et al., 1990; Green et al., 1991; Atkins et al., 1994; Facklam and Elliot, 1995; Sarma and Mohanty, 1998; Barton et al., 2001]). The sources of pediococci in the human body appear to be the enteral tract (Ruoff et al., 1988; Riebel and Washington, 1990), although food has also been implicated as a possible source for transmission of pediococci (Mastro et al., 1990). To date, however, there are no data which unequivocally show that the pediococci contained in food and which are used for bioprocessing or biopreservation have been implicated in causing disease. Green et al. (1991) monitored the fecal colonization and infection by vancomycin-resistant Gram-positive cocci in children who have undergone liver transplantation. These authors found an almost linear increase in percentage of colonized patients during the first month after transplantation, suggesting a nosocomial acquisition of the bacteria (Green et al., 1991). Thus, in the case of the study by Green et al. (1991), it appears that the indigenous flora of the pediatric liver transplant patients were not a source of colonizing bacteria.

Pediococcus acidilactici strains appear to be the most frequently isolated pediococci associated with infections, followed by *P. pentosaceus* and *P. urinae-equi* (Facklam and Elliot, 1995; Barros et al., 2001). *Pediococcus acidilactici* were isolated about five times more frequently than *P. pentosaceus* in a study by Facklam et al. (1989). All pediococci share the characteristic that they have intrinsic and high level resistance to vancomycin and teicoplanin (Swenson et al., 1990; Tankovic et al., 1993) and are thus often isolated after vancomycin treatment, together with other vancomycin-resistant LAB (Colman and Efstratiou, 1987; Ruoff et al., 1988; Golledge, 1990; Mastro et al., 1990; Swenson et al., 1990; Green et al., 1991). In addition, pediococci appear to be resistant to quinolone antibiotics as well as tetracycline (Tankovic et al., 1993; Sarma and Mohanty, 1998). They are generally sensitive towards antibiotics such as penicillin, ampicillin and aminoglycosides (especially gentamicin and netilmicin) and moderately sensitive towards chloramphenicol (Mastro et al., 1990; Swenson et al., 1990; Tankovic et al., 1993; Barton et al., 2001). The majority of strains appear to be sensitive also to erythromycin (Riebel and Washington, 1990; Tankovic et al., 1993). Thus, a variety of antibiotics can be used to treat pediococcal infections, once the *Pediococcus* strains have been identified as causative agents. However, little is known about the virulence factors of the pediococci. Clearly, pediococci are not potent pathogens because they are typical opportunists infecting only persons debilitated by major underlying disease. The antibiotic resistance of pediococci may help these bacteria to persist in the hospital environment and give them a competitive advantage upon infection. Some pediococci are also known to be α-hemolytic (Riebel and Washington, 1990), but the involvement of this trait in disease has not been demonstrated. Clearly therefore, the virulence traits which allow pediococci to invade and persist in the human host are largely unknown and require further investigation.

BIOTECHNICAL APPLICATIONS   The pediococci have had considerable biotechnical impact because of the practical application of pediocin-producing strains (see above) as biopreservative agents and the important role that pediococci have traditionally played as starter cultures for fermented foods, especially meats and vegetables (Luchansky et al., 1992; Mattila-Sandholm et al., 1993; Knorr, 1998). Furthermore, because pediococci are good lactate producers, they have an important role as silage starters in the production of animal feed. Pediococci have also been used or investigated for use as probiotics for humans or animals, although the use of *Bifidobacterium* or *Lactobacillus* spp. as probiotics has received far greater attention. *Tetragenococcus halophilus* is known for its tolerance to high levels (>18% NaCl) of salt and its association with the manufacture of soy sauce, miso, and fermented or salted fish products (Villar et al., 1985; Simpson and Taguchi, 1995; Tanasupawat et al., 2002; Kobayashi et al., 2003). Like *T. halophilus*, *T. muriaticus* is tolerant to high levels of salt and has been isolated mainly from fermenting fish products (Satomi et al., 1997; Kobayashi et al., 2000; Kobayashi et al., 2003). Some of the bio-

technical applications of the pediococci are summarized in Table 5.

MEAT FERMENTATION *Pediococcus* strains are used in the production of semi-dry fermented sausages, especially in North America, whereas in Europe the use of *L. sakei* and *L. curvatus* strains are preferred (Bacus and Brown, 1981; Raccach, 1987; Kang and Fung, 1999; Leroy and DeVuyst, 2004). *Pediococcus* has been used in the United States since the late 1950s as starter culture for the production of sausages like summer sausage, and initially the use of *P. pentosaceus* was recommended (Deibel and Niven, 1957; Deibel et al., 1961). Presently, the starter culture used varies and depends on the fermentation temperature and the desired final pH of the product. For high temperature fermentations and products with low pH, *P. acidilactici* strains are preferred, whereas *P. penstosaceus* is used either for low or high temperature fermentations (Ray, 2001). For the dry sausages fermentation, *P. pentosaceus* is better suited as it has a lower optimum growth temperature and a lower minimum temperature for fermentation (Raccach, 1987). The pediococci produce sufficient quantities of lactic acid via homofermentation to enhance gel formation in the meat as well as improve its flavor (Liepe, 1983). The ability of pediococci to grow and metabolize under conditions of reduced a_w during sausage manufacture favors the growth of pediococci in this matrix. In addition, these bacteria improve sausage uniformity and reduce process times (Everson et al., 1970).

VEGETABLE FERMENTATION Pediococci are also well known to play a role in vegetable fermentations. Natural starter cultures for the preparation of sauerkraut consist mainly of *Leuconostoc* spp. but also, to a lesser extent, *Lactobacillus* and *Pediococcus* spp. (Coster and White, 1964; Back, 1978b; Stamer, 1983; Ray, 2001). Usually these appear in succession, with *L. mesenteroides* starting off the fermentation, followed by *L. brevis*, *P. pentosaceus* and finally *L. plantarum* (Ray, 2001). Pediococci are also associated with cucumber (Stamer, 1983) and olive fermentation (Fernández et al., 1995). In the latter fermentation, they are usually found at the end of the first stage or the beginning of the second phase of fermentation, thus replacing a predominantly Gram-negative flora in the first stage and starting the lactic fermentation in the second stage. They account for about 35% of the isolated species of the Gram-positive cocci during the second phase of the fermentation and are eventually replaced by the lactobacilli in the third phase of the fermentation (Fernández et al., 1995). Pediococci also are part of the natural flora of African fermented foods such as hussuwa made from sorghum (Yousif et al., 2004) and togwa made from maize and sorghum (Mugula et al., 2003). They also play a role in the acidification of malt mash and wort in the brewery (Back, 1994; Back, 2000).

DAIRY FERMENTATIONS The role of pediococci in fermented dairy products is not as important as that of *Streptococcus thermophilus*, *Lactococcus lactis* or certain *Leuconostoc* or *Lactobacillus* strains. Pediococci usually belong to the non-starter lactic acid bacteria (NSLAB). These are adventitious bacteria that come from the environment during milking and contaminate the milk or the cheese curd (Ogier et al., 2002). Thus, they represent only a small proportion of the total LAB in cheese (Litopoulou-Tzanetaki et al., 1989; Simpson and Taguchi, 1995; Ogier et al., 2002) and develop during cheese maturation (Law et al., 1976; Simitsopoulou et al., 1997). Pediococci have been isolated as NSLAB from cheeses such as Comté (Bouton et al., 1998), Salers cheese (Callon et al., 2004), and Parmigiano Reggiano (Coppola et al., 1997), where they may facilitate the cheese making process via their proteolytic, lipolytic and esterolytic activities (Bhowmik and Marth, 1990a; El-Soda et al., 1994; Vafopoulou-Mastrojiannaki et al., 1994; Bouton et al., 1998). *Pediococcus pentosaceus* strains from cheese preferentially hydrolyzed β-casein and had high proteinase, aminopeptidase, and dipeptidyl aminopeptidase activities, while exhibiting low esterase activity (Vafopoulou-Mastrojiannaki et al., 1994). *Pediococcus* spp. have also been used as adjunct starter cultures in cheese production, but their influence on cheese quality is not always fully understood (Dacre, 1958; Reinbold and Reddy, 1978; Bhowmik et al., 1990c; Ohlson, 1990; Simpson and Taguchi, 1995). Caldwell et al. (1998) genetically modified *Pediococcus* spp. to construct lactose-positive strains, which may be used as alternatives to *Streptococcus thermophilus* strains as starters in the manufacture of Mozzarella. Although pediococci are normally not good starter cultures to use in milk as these cells are lactose-negative, Caldwell et al. (1998) showed that genetic modification could result in successful alternative starter cultures for Mozzarella production. Because bacteriophage attack on *S. thermophilus* strains used in Mozzarella manufacture is increasingly becoming a problem (Caldwell et al., 1998), there is a need for alternatives such as this genetically modified starter in Mozzarella production.

SILAGE FERMENTATION Conservation of forage crops by ensiling is based on natural fermentation in which LAB convert sugars to lactic acid

under anaerobic conditions. As a result the pH decreases and the forage is conserved (McDonald et al., 1991). In recent years much attention was focused on the use of homofermentative LAB as silage inoculants. However, not all starter cultures appear to be suitable for all kinds of forages nor for a variety of environmental conditions (Zhang et al., 2000). Generally, isolates that occur naturally on the forage crops are tested as starters (Cai et al., 1999; Zhang et al., 2000). Pediococci occur naturally on various types of forage crops (Cai et al., 1999; Zhang et al., 2000) and Zhang et al. (2000) showed that for perennial ryegrass silage *P. acidilactici* and *P. pentosaceus* strains had beneficial effects on the silage fermentation quality. Similarly, Cai et al. (1999) showed that *P. acidilactici* and *P. pentosaceus* were suitable starters for the ensiling alfalfa and Italian ryegrass silage. Fitzsimons et al. (1992) showed in laboratory ensilage models that a *P. acidilactici* strain showed favorable characteristics as a silage inoculant. Interestingly, in the studies of Cai et al. (1999) and Zhang et al. (2000) the pediococci, especially *P. acidilactici* strains, were particularly heat tolerant when compared to other homofermentative LAB and thus successful for ensiling also at high temperatures. The use of *P. acidilactici* strains as inoculants would thus be preferred in countries with high ambient temperatures, in which the temperatures during early ensilage may exceed 45°C (Zhang et al., 2000).

BIOPRESERVATION OF FOODS Production of pediocins by pediococci (see above) has been intensively investigated in view of use of either the purified bacteriocins or the producer cultures in biopreservation of foods. Pediococins produced by *P. acidilactici* or *P. pentosaceus* strains include pediocin AcH (Bhunia et al., 1988; Biswas et al., 1991), pediocin PA-1 (Gonzalez and Kunka, 1987; Pucci et al., 1988), pediocin SJ-1 (Schved et al., 1993), pediocin A (Piva and Headon, 1994), pediocin Np5 (Manca de Nadra, 1998), pediocin PO_2 (Coventry et al., 1995), pediocin PD-1 (Green et al., 1997), and pediocin ACCEL (Wu et al., 2004). The use of pediocin, mostly pediocin AcH (same as PA-1), for biopreservation of especially meats has been investigated in laboratory-type, model studies. In studies with dry sausage fermentation, the use of bacteriocin-producing *P. acidilactici* JD1-23, *P. acidilactici* PAC 1.0 as fermenting agents resulted in numbers of *L. monocytogenes* per gram of dry sausage that were 1–2 logs lower than that in control sausages (Berry et al., 1990; Foegeding et al., 1992; Luchansky et al., 1992; Baccus-Taylor et al., 1993; Typpöen et al., 2003). Pediocin PA-1 was also experimentally used as a dried powder preparation to successfully inhibit *L. monocyto-*

genes in food systems such as dressed cottage cheese, half-and-half cream, and cheese sauce (Pucci et al., 1988). Two commercial antimicrobial compounds which have found use in the food industry and which are licensed for use are Microgard and Alta 2341. These are not purified bacteriocin compounds but rather ferments of pediocin-producing bacteria that impart antibacterial properties to the foods (Stiles, 1996).

PEDIOCOCCI USED AS PROBIOTICS Pediococci, compared to other *Lactobacillus* and *Pediococcus* spp., are used relatively rarely as probiotics. Geary et al. (1999) showed that fermentation of a liquid diet with *P. acidilactici* for feeding newly weaned pigs could effectively reduce the pH of the diet to below 4.0, and could also eliminate enteropathogens and spoilage organisms from the diet. Weese and Arroyo (2003) performed a bacteriological evaluation of cat and dog foods claiming to contain probiotics. They found eleven of nineteen diets contained additional, related organisms, including *Pediococcus* spp. in four products (Weese and Arroyo, 2003). However, these were not indicated on the product label, and it is unclear whether these pediococci were added intentionally or whether they represented contaminants.

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CHAPTER 1.2.9

Genera *Leuconostoc*, *Oenococcus* and *Weissella*

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Introduction

The genera *Leuconostoc*, *Oenococcus* and *Weissella*—typical lactic acid bacteria (LAB)—geno- and phenotypically most closely resemble the genus *Lactobacillus* in that all representatives are Gram-positive, catalase-negative, and facultatively anaerobic. The genus *Weissella* in fact harbors two different morphological types: the rods (formerly the "atypical" heterofermentative lactobacilli) and the ovoid-shaped cocci (typical also of the *leuconostocs*, *Oenococcus* and *streptococci*) *Weissella paramesenteroides* and *Weissella hellenica*. However, a third ovoid-shaped *Weissella* species (*Weissella thailandensis*), isolated from fermented fish in Thailand, has recently been described (Tanasupawat et al., 2000). Together with *Pediococcus*, these genera share physiological relatedness and have in fact already at an earlier stage been considered to be phylogenetically "intermixed" (Stackebrandt and Teuber, 1988). *Leuconostoc paramesenteroides* was shown to form a natural phylogenetic group with the former lactobacilli *Lactobacillus confusus*, *Lactobacillus halotolerans*, *Lactobacillus kandleri*, *Lactobacillus minor* and *Lactobacillus viridescens*, on the basis both of comparative 16S rRNA sequence analysis (Yang and Woese, 1989) and 23S rRNA similarity mapping (Schillinger et al., 1989). These species have now been reclassified as *Weissella* spp. (Collins et al., 1993), while *Leuconostoc oenos* has been assigned to the new genus *Oenococcus* (Dicks et al., 1995c). The major physiological features for species of the genera *Leuconostoc*, *Oenococcus* and *Weissella* are presented in Table 1.

Physiological characteristics such as the absence of arginine deiminase, and the production of predominantly D(–)-lactate from glucose, are shared by all *Leuconostoc* species and *Oenococcus*, and thus far, only by the ovoid-shaped weissellas (*W. paramesenteroides*, *W. hellenica* and *W. thailandensis*). Among the rod-shaped weissellas, arginine-dihydrolase is absent only in *Weissella viridescens* and in a few obligately heterofermentative lactobacilli (*Lactobacillus oris*, *Lactobacillus vaccinostercus*, *Lactobacillus san-* *franciscensis* and *Lactobacillus fructosus*; Hammer and Vogel, 1995). Likewise, only some heterofermentative lactobacilli, which do not exhibit the typical peptidoglycan interpeptide bridge (D-isoasparagine) of lactobacilli, have been transferred from the genus *Lactobacillus* to *Weissella*. However, *Lactobacillus suebicus*, with the diaminopimelic acid (m-A$_2$pm) peptidoglycan type, *Lactobacillus vaccinostercus* (m-A$_2$pm), *Lactobacillus sanfranciscensis* (Lys-Ala), and *Lactobacillus fructosus* (Lys-Ala) remain in the genus *Lactobacillus*.

Presently, the genus *Leuconostoc* comprises 10 species, with 3 subspecies recognized for *Leuconostoc mesenteroides*. The genus *Weissella* comprises 8 species; a ninth species (*Weissella cibaria*) has been proposed for strains isolated from Malaysian foods and clinical samples (Björkroth et al., 2002). The genus *Oenococcus* is thus far represented by only one species, *Oenococcus oeni*, which has been shown by Zavaleta et al. (1997b) to be phylogenetically homogeneous on the basis of 16S-23S-rDNA intergenic sequence analysis.

Phylogeny

The knowledge of bacterial phylogeny is mainly based on sequence analysis of 16S rRNA (Woese, 1987). Also other macromolecules, such as 23S rRNA (Ludwig and Schleifer, 1994), elongation factor Tu, and the β-subunit of ATPase (Ludwig et al., 1993), have been used for phylogenetic studies of Gram-positive bacteria. These studies show that the genera of *Leuconostoc* and *Weissella* and the species *Oenococcus oeni* belong to the so-called "*Clostridium* branch." This line of descent (phylum) consists of Gram-positive organisms having guanine plus cytosine (G+C) DNA content of less than 50 mol%. Also, the other genera of typical LAB, such as *Lactobacillus*, *Lactococcus*, *Enterococcus* and *Carnobacterium* belong to this *Clostridium* branch.

Phylogenetically, *Leuconostoc* and the related genera *Weissella* and *Oenococcus* are well

Table 1. Differentiation of *Leuconostoc* and *Weissella* from other lactic acid bacteria.

	Leuconostoc	Weissella	Heteroferm. lactobacilli	Homoferm. lactobacilli	Enterococcus Lactococcus Streptococcus	Pediococcus	Carnobacterium
Morphology	Ovoid	Ovoid or rods	Rods	Rods	Ovoid	Cocci in tetrads / pairs	Rods
Gas from glucose	+	+	+	–	–	–	(+)
Hydrolysis of arginine	–	±	±	–/+	– or +	– or +	+
Dextran from sucrose	–/+	–/+	–/+	–/+	–/+	–	–
Isomer of lactic acid from glucose	D(–)	D(–) or DL	DL	D(–), DL, or L(+)	L(+)	DL or L(+)	L(+)
Peptidoglycan	Lys-Ala/Ser[a]	Lys-Ala/Ser[a]	Lys-Asp and others[b]	Lys-Asp/m-A$_2$pm and others[b]	Lys-Asp	Lys-Asp	m-A$_2$pm

Symbols: +, positive reaction; (+), weak positive reaction; ±, mostly positive, only a few species negative; and –/+, mostly negative, only a few species positive.
Abbreviations: Lys, lysine; Ala, alanine; Ser, serine; Asp, aspartate; and m-A$_2$pm, 2,6-diaminopimelic acid (2,6-diaminoheptanedioic acid).
[a]No Lys-Asp-types, but different variations of Lys-Ala/Ser types (exception *W. kandleri*).
[b]Related to those of *Leuconostoc* and *Weissella*. From Garvie (1984) and Shaw and Harding (1984).
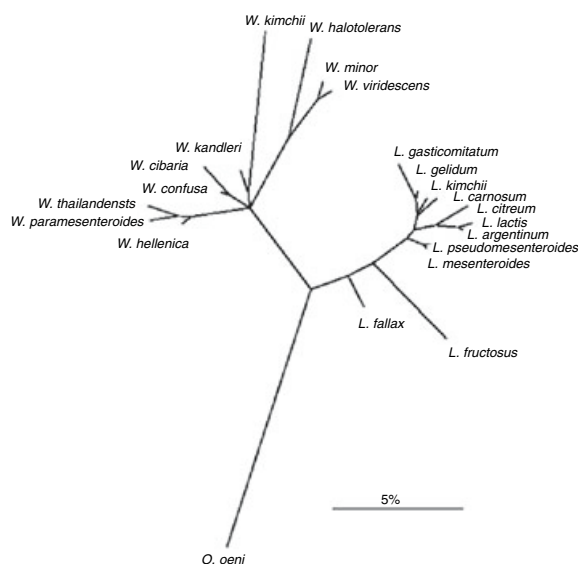
Fig. 1. 16S rRNA-based tree reflecting the relationships of *Leuconostoc*, *Oenococcus* and *Weissella* species. The tree was reconstructed applying a maximum parsimony approach upon a dataset comprising more than 20,000 almost complete small subunit rRNA sequences from representatives of all three domains. The topology was evaluated and corrected according to the results obtained by using alternative treeing methods (distance matrix and maximum likelihood). Multifurcations indicate that a common relative branching order could not unambiguously be determined or was not supported applying the different methods. Only alignment positions at which at least 50% of all available sequences from representatives of the *Leuconostoc*, *Oenococcus*, *Weissella* cluster share common nucleotides were included for the calculations. The bar indicates 5% estimated base changes.

separated from the other LAB (Olsen et al., 1991; De Rijk et al., 1992; see Fig. 1). Both 16S (Martinez-Murcia and Collins, 1990; Martinez-Murcia and Collins, 1991; Collins et al., 1993) and 23S rRNA (Martinez-Murcia et al., 1993) analyses have shown that the *Leuconostoc* group comprises actually three distinct evolutionary lines. These evolutionary lines have been called the *Leuconostoc sensu stricto*, *Leuconostoc paramesenteroides* and *Leuconostoc oenos* lines, respectively. The discovery of these lines was the major reason for two reclassification proposals. Currently, the genus *Weissella* is associated with the evolutionary line of *Leuconostoc paramesenteroides*, and *Oenococcus oeni* with the *Leuconostoc oenos* line. The reclassification of *L. paramesenteroides* and the related *Lactobacillus* species under a new genus *Weissella* was proposed in 1993 by Collins et al. (1993). Two years later, Dicks et al. (1995) suggested that *Leuconostoc oenos* should be reclassified as *Oenococcus oeni* gen. nov., comb. nov.

Phylogeny of the *Leuconostoc* Species

The *Leuconostoc sensu stricto* species *L. argentinum*, *L. carnosum*, *L. citreum*, *L. gasicomitatum*, *L. gelidum*, *L. kimchii*, *L. lactis*, *L. mesenteroides* (three subspecies: *cremoris*, *dextranicum* and *mesenteroides*) and *L. pseudomesenteroides* show 97.1–99.5% 16S rRNA sequence similarity (Table 2). In addition to these species, an atypical *Leuconostoc* species, *L. fallax* has been described (Martinez-Murcia and Collins, 1991). It possesses 94–95% 16S rRNA similarity with the other leuconostocs representing a peripheral line within the genus *Leuconostoc*. In Fig. 2, the morphology of *L. gasicomitatum* is shown.

When the phylogeny of *Leuconostoc sensu stricto* is placed under more precise scrutiny, three evolutionary branches can be distinguished further. One contains *L. carnosum*, *L. gasicomitatum*, and *L. gelidum*, another *L. citreum* and *L. lactis* and the third *L. mesenteroides* and *L. pseudomesenteroides*. The sequence data of *L. argentinum* has only lately been deposited in the National Centre for Biotechnology Information (NCBI) sequence database (Entrez: accession AF175403+). Based on this sequence, *L. argentinum* joins the *L. citreum-L. lactis* line.

Phylogeny of the *Weissella* Species

The phylogenetic status of the current genus *Weissella* was clarified in 1990. Using both 16S and 23S rRNA sequence data, Martinez-Murcia and Collins (1990) showed that *L. paramesenteroides* is phylogenetically distinct from *L. mesenteroides* and that it forms a natural grouping with certain heterofermentative *Lactobacillus* species. These lactobacilli included *L. confusus*, *L. kandleri*, *L. minor* and *L. viridescens*. In a study of leuconostoc-like organisms originating from fermented sausages, the phylogenetic status of these organisms was further assessed (Collins et al., 1993). This resulted in the establishment of a new genus *Weissella* in which *Leuconostoc paramesenteroides*, the three former *Lactobacillus* species and a new species *W. hellenica* were assigned (see Table 3).

Currently, 8 species are recognized in this genus, i.e., *W. confusa*, *W. halotolerans*, *W. hellenica*, *W. kandleri*, *W. minor*, *W. paramesenteroides*, *W. thailandensis* and *W. viridescens* possessing 93.9–99.1% 16S sequence similarity (Table 4). The phylogenetic lines within the genus *Weissella* are differentiated into four main branches. One of the branches contains *W. hellenica*, *W. paramesenteroides* and *W. thailandensis*. The type strains of these three species possess 96.8–98.6% 16S rRNA similarity (Table 4). *Weissella confusa* and and *W. cibaria* represent a second lineage. *Weissella minor*, *W. viridescens* and *W. halotolerans*

Table 2. Percentage sequence similarities for a 1491-nucleotide region of 16S rRNA of the current species in the genus *Leuconostoc* and of *Weissella paramesenteroides*.

Species	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
1. *L. argentinum* LMG 18534[T]	100	97.5	98.7	92.4	97.1	99.3	97.7	97.5	97.8	90.3
2. *L. carnosum* LMG 11498[T]	97.5	100	97.4	94.3	98.3	97.3	97.8	97.8	97.8	91.0
3. *L. citreum* LMG 9824[T]	98.7	97.4	100	94.5	97.4	98.5	07.7	97.7	97.5	91.2
4. *L. fallax* LMG 13177[T]	92.4	94.3	94.5	100	93.5	93.6	94.6	94.6	94.6	92.4
5. *L. gelidum* LMG 9850[T]	97.1	98.3	97.4	93.5	100	97.6	97.9	97.9	98.0	90.9
6. *L. lactis* LMG 8894[T]	99.3	97.3	98.5	93.6	97.6	100	98.2	98.2	98.0	91.3
7. *L. mesenteroides* subsp. *cremoris* LMG 6909[T]	97.7	97.8	97.7	94.6	97.9	98.2	100	100	99.5	91.8
8. *L. mesenteroides* subsp. *mesenteroides* LMG 6893[T]	97.5	97.8	97.7	94.6	97.9	98.2	100	100	99.5	91.8
9. *L. pseudomesenteroides* LMG 11482[T]	97.8	97.8	97.5	94.6	98.0	98.0	99.5	99.5	100	91.7
10. *W. paramesenteroides* LMG 9852[T]	90.3	91.0	91.2	92.4	90.9	91.3	91.8	91.8	91.7	100

Abbreviation: [T], type strain.
[a]The sequence data used for constructing this table originates from the National Centre for Biotechnology Information Entrez database.

form another line possessing 96.8–99.1% 16S rRNA similarity (Table 4). Apart from these three is the fourth line comprising *W. kandleri*. The morphology of *W. cibaria* is depicted in Fig. 3.

Oenococcus oeni

The reclassification of *Leuconostoc oenos* (Garvie, 1967a) as *Oenococcus oeni* gen. nov.,
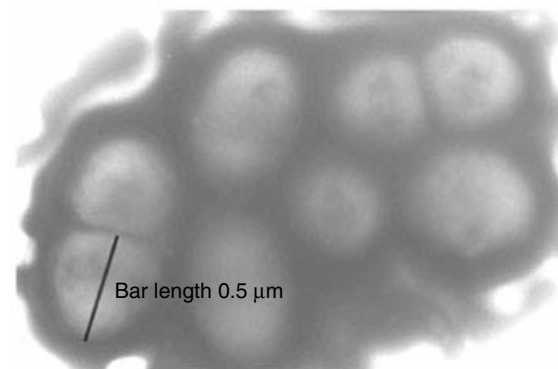
Fig. 2. *Leuconostoc gasicomitatum* type strain LMG 18811. This transmission electron micrograph shows cells that have been resuspended in physiological NaCl, negatively stained with 1% of phosphotungstic acid, and examined using a JEOL JEM 100 electron microscope. Courtesy of Professor Antti Sukura, Department of Pathology, Faculty of Veterinary Medicine, University of Helsinki.

comb. nov., was proposed by Dicks et al. (1995b). Already in 1993 Martinez-Murcia et al. (1993) showed by comparison of both 16S and 23S rRNA sequences that *L. oenos* does not belong to the same line of descent with the *Leuconostoc sensu stricto* organisms or *Leuconostoc paramesenteroides* group of species (the current genus *Weissella*).

Related to the phylogeny of *Oenococcus oeni*, an interesting debate over its evolution speed has occurred. *Oenococcus oeni* was considered to be a tachytelic (fast-evolving) organism on the basis of its 16S rRNA sequences (Yang and Woese, 1989). The phylogenetic tree of *O. oeni* has a very long branch, compared to its adjacent neighbors, the *Leuconostoc sensu stricto* organisms and *Weissella*, and the 16S rRNA of *O. oeni* varies in otherwise conserved positions. These traits are considered to be indications of a fast-evolving nature. However, a study employing *rpoC* gene sequences (Morse et al., 1996) does not support this theory.

Taxonomy

During the last 10 years, two major changes have occurred in the taxonomy of *Leuconostoc* and related organisms. A new genus *Weissella* has been proposed to cover the former *Leuconostoc*

Table 3. Assignment of former *Lactobacillus* or *Leuconostoc* species to the current status as species of the genus *Weissella*.

Former designation as *Lactobacillus* or *Leuconostoc*	Current designation as *Weissella*[a]
Leuconostoc paramesenteroides	*W. paramesenteroides*
Lactobacillus confuses	*W. confusa*
Lactobacillus halotolerans	*W. halotolerans*
Lactobacillus kandleri	*W. kandleri*
Lactobacillus minor	*W. minor*
Lactobacillus viridescens	*W. viridescens*
NF	*W. hellenica* sp. nov.
NF	*W. thailandensis* sp. nov.

Abbreviation: NF, no former assignment.
[a]From Collins et al. (1993).
Data compiled from Collins et al. (1993, 1994) and Tanasupawat et al. (2000).

Table 4. Percentage sequence similarities for a 1340-nucleotide region of 16S rRNA of the current species in the genus *Weissella* and of *Leuconostoc mesenteroides*.

Species	1.	2.	3.	4.	5.	6.	7.	8.
1. *W. confusa* NCIMB 9311[T]	100	94.9	97.2	96.8	96.8	96.8	97.0	91.5
2. *W. halotolerans* DSM 20190[T]	94.9	100	94.3	95.6	96.6	93.9	96.6	88.7
3. *W. hellenica* NCFB 2973[T]	97.2	94.3	100	95.5	96.3	98.6	96.4	90.2
4. *W. kandleri* NCFB 2753[T]	96.8	95.6	95.5	100	96.5	95.3	96.7	90.5
5. *W. minor* NCFB 1973[T]	96.8	96.6	96.3	96.5	100	95.8	99.1	90.2
6. *W. paramesenteroides* DSM 20288[T]	96.8	93.9	98.6	95.3	95.8	100	95.8	91.1
7. *W. viridescens* NCIMB 8965[T]	97.0	96.6	96.4	96.7	99.1	95.8	100	89.9
8. *L. mesenteroides* subsp. *mesenteroides* NCIMB 8023[T]	91.5	88.7	90.2	90.5	90.2	91.1	89.9	100

Abbreviation: [T], Type strain.
From Collins (1993).
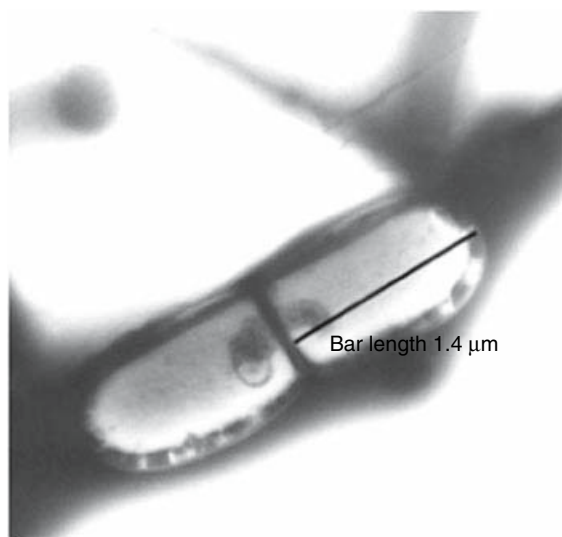
Fig. 3. *Weissella cibaria* type strain LMG 17699. This transmission electron micrograph shows cells that have been resuspended in physiological NaCl, negatively stained with 1% of phosphotungstic acid, and examined using a JEOL JEM 100 electron microscope. Courtesy of Professor Antti Sukura, Department of Pathology, Faculty of Veterinary Medicine, University of Helsinki.

paramesenteroides group (Collins et al., 1993), and some "atypical" heterofermentative lactobacilli. *Leuconostoc oenos* (Garvie, 1967a) has been reclassified as *Oenococcus oeni* gen. nov., comb. nov. (Dicks et al., 1995c).

Species of the Genus *Leuconostoc*

Currently, 10 species of the genus *Leuconostoc* are recognized. They are *L. argentinum* (Dicks et al., 1993), *L. carnosum* (Shaw and Harding, 1989), *L. citreum* (Farrow et al., 1989), *L. fallax* (Martinez-Murcia and Collins, 1991), *L. gasicomitatum* (Björkroth et al., 2002), *L. gelidum* (Shaw and Harding, 1989), *L. kimchi* (Kim et al., 2000a), *L. lactis* (Garvie, 1960), *L. mesenteroides* (Garvie, 1979; Garvie, 1983) and *L. pseudomesenteroides* (Farrow et al., 1989). The former species *L. cremoris* and *L. dextranicum* are presently considered as two subspecies of *L. mesenteroides* together with the third subspecies *mesenteroides* (Garvie, 1983). In 1992, *L. amelibiosum* was found to be synonymous with *L. citreum* and the species designation "amelibiosum" was dropped (Takashi et al., 1992).

These 10 species belong to the *Leuconostoc sensu stricto* line of descent. Classical identification schemes for leuconostocs are usually based on the following phenotypic characteristics:

Gram-positive, coccoid to ovoid-like morphology, catalase negative, arginine negative, gas production from glucose, and production of primarily the D(−)-lactate isomer. Classification to the species level within the genus *Leuconostoc* is problematic by means of phenotypic testing. Therefore, many approaches employing different macromolecules and numerical analysis have been introduced.

Species of the Genus *Weissella*

Currently (2002), the genus *Weissella* consists of the following 8 species: *W. confusa* (Holzapfel and Kandler, 1969b), *W. halotolerans* (Kandler et al., 1983), *W. hellenica* (Collins et al., 1993), *W. kandleri* (Holzapfel and van Wyk, 1982), *W. minor* (Kandler et al., 1983), *W. paramesenteroides* (Garvie, 1967b), *W. thailandensis* (Tanasupawat et al., 2000), and *W. viridescens* (Niven and Evans, 1957; Kandler and Abo-Elnaga, 1966). With the exception of *W. hellenica* and *W. thailandensis*, these species were formerly classified in the genera *Lactobacillus* or *Leuconostoc*. Table 4 shows those species currently assigned to *Weissella*. A ninth species of *Weissella*, *Weissella cibaria*, has been suggested (Björkroth et al., 2002). This species comprises strains isolated from traditional Malaysian foods and clinical samples.

Phylogenetically, weissellas belong to those species previously called "the *Leuconostoc paramesenteroides* group" (Schillinger et al., 1989). The taxonomic status of these organisms was clarified by the means of phylogenetic methods (Martinez-Murcia and Collins, 1991; Collins et al., 1993) and approaches using numerical analysis of whole-cell protein profiles.

The morphology of weissellas varies from spherical or lenticular cells to irregular rods. The former *Leuconostoc* species, *W. hellenica* and *W. paramesenteroides*, produce the D(−)-lactate isomer during the fermentation of glucose, in contrast to the other *Weissella* species, which produce DL-lactate. Another feature of the weissellas, being different from the constant phenotypic trait of leuconostocs, is the variability in the production of ammonia from arginine. *Leuconostoc* spp. are all arginine-negative, whereas *W. cibaria*, *W. confusa*, *W. halotolerans*, *W. kandleri* and *W. minor* are arginine positive. *Weissella viridescens*, *W. paramesenteroides* and *W. hellenica* do not hydrolyze arginine.

Oenococcus oeni

Oenococcus oeni is the only species in the genus *Oenococcus*. The reclassification of this former

Leuconostoc oenos (Garvie, 1967a) as *O. oeni* gen. nov., comb. nov. was proposed by Dicks et al. (1995c). This proposal was due to the results obtained from phylogenetic, phenotypic and genotypic studies.

Compared to leuconostocs and weissellas, *O. oeni* is acidophilic, growing at an initial pH of 4.8. Its habitat is wine and related habitats; therefore it tolerates ethanol and grows in media containing 10% ethanol. Phenotypic characteristics distinguishing *O. oeni* from leuconostocs and weissellas are listed in Table 5.

DNA-DNA or DNA-rRNA Reassociation Levels

Different reassociation techniques have been used to clarify the taxonomy of *Leuconostoc*, *Weissella* and *Oenococcus oeni*. Hontebeyrie and Gasser (1977) used the hydroxylapatite method of Brenner (1973). Garvie (1976), and Vescovo et al. (1979), and Farrow et al. (1989) applied a membrane filter method (Denhardt, 1966), whereas Schillinger et al. (1989) used both the filter method and an optical method of De Ley et al. (1970). Shaw and Harding (1989) used the S1 nuclease procedure of Crosa et al. (1973) in their taxonomic studies. The results obtained by these different methods are in good agreement. Table 6 lists the DNA-DNA similarity values for leuconostocs and the related organisms.

Only a few studies of LAB have been done by the means of DNA-rRNA hybridizations. Results from the studies performed by Garvie (1981) and Schillinger (1989) confirmed the distant relationship between *O. oeni* and *Leuconostoc*.

Habitats

Leuconostocs and weissellas are obligately fermentative chemoorganotrophs. They are fastidious in their nutrient requirements, and share numerous natural and artificial habitats (especially food substrates) with other LAB, and particularly with the lactobacilli, pediococci and carnobacteria. Growth factors such as amino acids and peptides, fermentable carbohydrates, fatty acids, nucleic acids and particular vitamins, support their nutritional requirements. Practically all strains seem to require biotin, nicotine, thiamine, and pantothenic acids or its derivatives. Energy generation is by carbohydrate fermentation via both the hexose-monophosphate and the phosphoketolase pathways, in combination with substrate-level phosphorylation. End products of glucose fermentation are CO_2, ethanol and/or acetate, and either D(–)-lactate (for all leuconostocs, *O. oeni*, and the ovoid-shaped

weissellas), or DL-lactate (for all rod-shaped strains). Reports on the association of leuconostocs and weissellas with man and animals are relatively rare and mainly refer to the digestive tract. In the small and large intestines, the numbers of leuconostocs may range around 10^5/g. *Leuconostoc* and *Weissella* spp. were detected together with *Lactobacillus* and *Pediococcus* spp. in human feces by using group-specific polymerase chain reaction (PCR) primers and denaturing gradient gel electrophoresis of DNA fragments generated by PCR with 16S ribosomal DNA-targeted group-specific primers (Hertel et al., 2001). *Weissella viridescens* has been isolated from the stomach of the honey bee (Hammes et al., 1992), whereas *W. confusa* seems to be associated with the digestive tract of the frog (Tina, 1987). Strains of *Oenococcus oeni* require more restrictive growth conditions than most other LAB. This characteristic is indicated by their acidophilic nature, their adaptation to the wine environment, their alcohol tolerance, and their requirement for special growth factors, e.g., those present in tomato juice (Garvie and Mabbitt, 1967c).

Foods of Animal Origin

GENUS *LEUCONOSTOC* IN MEAT AND MEAT PRODUCTS Lactic acid bacteria are the predominant spoilage organisms in vacuum- or modified-atmosphere-packaged chill-stored meat or meat products (Borch et al., 1996; Korkeala and Björkroth, 1997). Anaerobic circumstances together with refrigerated temperatures prohibit the growth of Gram-negative spoilage organisms. Since LAB are resistant to the inhibition of nitrite and smoke and are able to grow in relatively high sodium chloride concentrations (Castellani and Niven, 1955; Dodds and Collins-Thompson, 1984; Delaquis, 1988; Korkeala et al., 1992), they grow also in cooked vacuum or modified atmosphere packaged meat products (Korkeala and Björkroth, 1997). Representatives, both of *Leuconostoc* and *Weissella*, have been found associated with a wide variety of meat products, including fresh and vacuum packaged meat, poultry, as well as processed and fermented meat products (Reuter, 1975; Von Holy and Holzapfel, 1989; Holzapfel and Schillinger, 1992; Holzapfel, 1998).

Rapid developments in vacuum and modified gas atmosphere packaging, and in refrigeration technologies, have influenced the distribution pattern of fresh meat dramatically, resulting in the establishment of a novel food ecosystem, the refrigerated vacuum-packaged meat product. In this system, a spoilage pattern concomitant with a dominant psychrotolerant population of lactobacilli and leuconostocs has been reported

Table 5. Phenotypic characteristics and mol% G+C in the DNA of *Leuconostoc* and *Weissella* spp. and *Oenococcus oeni*.

Characteristics	L. mesenteroides ssp.			L. pseudomesenteroides	L. citreum	L. carnosum	L. gasicomitatum	L. gelidum	L. lactis	L. argentinum
	mesenteroides	dextranicum	cremoris							
Acid from										
Amygdalin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Arabinose	+	−	−	d	+	−	+	+	−	d
Arbutin	d	−	−	d	+	−	−	+	−	−
Cellulose	d	d	−	ND	ND	ND	ND	ND	−	ND
Cellobiose	d	−	−	d	d	d	+	+	−	d
Fructose	+	+	−	+	+	+	+	+	+	d
Galactose	+	d	+	d	−	−	d	−	+	+
Lactose	d	+	d	d	−	−	−	−	+	+
Maltose	+	+	−	+	+	−	+	d	+	+
Mannitol	d	−	−	−	d	−	−	−	−	d
Mannose	d	d	−	+	+	d	+	+	d	+
Melibiose	d	d	−	d	−	d	+	+	d	+
Raffinose	d	d	−	d	−	−	+	+	d	+
Ribose	d	+	−	+	−	d	+	d	−	−
Salicin	d	−	−	d	+	d	−	+	d	−
Sucrose	+	+	−	d	+	+	+	+	+	+
Trehalose	+	+	−	+	+	+	+	+	−	d
Xylose	d	d	−	+	−	−	−	+	−	d
Ammonia from arginine	−	−	−	−	−	−	−	−	−	−
Lactic acid configuration	D(−)	D(−)	D(−)	D(−)	D(−)	D(−)	D(−)	D(−)	D(−)	D(−)
Hydrolysis of esculin	+	+	−	d	+	d	+	+	−	−
Dextran production	+	+	−	ND	ND	+	+	+	−	−
Growth at pH 4.8	−	−	−	ND	ND	ND	ND	ND	−	ND
Requirement of TJF	−	−	−	−	−	−	−	−	−	−
Growth in 10% ethanol	−	−	−	ND	ND	ND	ND	ND	−	ND
NAD-dependent G6PDH present	+	+	+	ND	ND	ND	ND	ND	+	ND
Growth at 37°C	d	+	−	+	d	−	−	−	+	+
Peptidoglycan type[b]	Lys-L-Ser-L-Ala$_2$	Lys-L-Ser-L-Ala$_2$	Lys-L-Ser-L-Ala$_2$	Lys-L-Ser-L-Ala$_2$	Lys-L-Ala$_2$	Lys-L-Ala$_2$	Lys-L-Ala$_2$	Lys-L-Ala$_2$	Lys-L-Ala$_2$	Lys-L-Ala$_2$
Cell morphology	Coccoid to elongated cocci	Coccoid to elongated cocci	Coccoid to elongated cocci	Coccoid to elongated cocci	Coccoid to elongated cocci	Coccoid to elongated cocci	Coccoid to elongated cocci	Coccoid to elongated cocci	Coccoid to elongated cocci	Coccoid to elongated cocci
Mol% G+C in the DNA	37–39	37–40	38–40	38–41	38–40	39	37	37	43–45	40.5

Symbols and Abbreviations: +, 90% or more of strains positive; −, 90% or more of strains negative; d, 11–98% of strains positive; (), delayed reaction; ND, no data. D, 90% or more of the lactic acid is D(−); DL, more than 25% of the total lactic acid is L(+); TJF, tomato juice factor; and NAD-dependent G6PDH, nicotinamide adenine dinucleotide-dependent glucose-6-phosphate dehydrogenase.

[a]The new species *L. cibaria.* was suggested by Björkroth et al. (2002).

[b]The peptidoglycan type of *L. carnosum*, *L. gelidum* and *L. argentinum* was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig).

Data for *L. mesenteroides*, *Leuconostoc lactis*, and *Oenococcus oeni* are from Garvie (1986).

Data for *L. carnosum* and *L. gelidum* are from Shaw and Harding (1989), *L. pseudomesenteroides* from Farrow et al. (1989), for *L. citreum* from Farrow et al. (1989) and Schillinger et al. (1989), for *L. argentinum* from Dicks et al. (1993), for *L. fallax* from Martinez-Murcia and Collins (1991), and for *L. gasicomitatum* sp. nov. from Björkroth et al. (2000).

Data for *Weissella* spp. are from Collins et al. (1993), and Tanasupawat et al. (2000).

(Allen and Foster, 1960; Reuter, 1970b; Reuter, 1975; Reuter, 1981; Mol et al., 1971; Kitchell and Shaw, 1975; Seideman et al., 1976; Christopher et al., 1979; Egan and Shay, 1982; Hitchener et al., 1982). Formerly considered as one of the minor groups among the "meat lactic" bacteria, the leuconostocs have increasingly been reported as a major group in refrigerated, vacuum, or modified-atmosphere packaged meats during the past two decades (Hanna et al., 1981; Hanna et al., 1983; Savell et al., 1981; Savell et al., 1986; Shaw and Harding, 1984; Schillinger and Lücke, 1986),

L. fallax	L. kimchii	O. oeni	W. thailandensis	W. kandleri	W. viridescens	W. minor	W. halotolerans	W. confusa	W. paramesenteroides	W. hellenica	W. cibaria[a] nov.
ND	+	ND	−	−	−	−	ND	+	ND	ND	+
−	+	d	+	−	−	−	−	−	d	+	+
−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+
ND	ND	d	ND	ND	ND	ND	ND	ND	d	ND	ND
−	+	d	−	−	−	+	−	+	(d)	−	+
+	+	+	+	+	+	+	+	+	+	+	+
−	+	d	+	+	−	−	−	+	+	−	−
−	+	−	−	ND	ND	ND	ND	ND	ND	ND	−
+	+	−	+	−	+	+	+	+	+	+	+
(d)	+	−	−	−	−	−	−	d	ND	ND	−
+	+	d	+	−	+	+	+	+	+	ND	+
−	−	d	+	−	−	−	−	−	+	−	−
−	−	−	+	−	−	−	−	−	D	−	−
+	+	d	+	+	−	+	+	+	ND	−	−
−	+	d	−	ND	ND	ND	ND	ND	ND	ND	+
+	+	−	+	−	d	+	−	+	+	+	+
(d)	+	+	+	−	d	+	−	−	+	+	−
−	−	d	−	−	−	−	−	+	d	−	+
−	−	−	−	+	−	+	+	+	−	−	+
D(−)	D(−)	D(−)	D(−)	DL	DL	DL	DL	DL	D(−)	D(−)	DL
ND	ND	+	−	−	−	+	−	+	ND	ND	+
ND	+	−	−	+	ND	−	ND	+	−	−	+
ND	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	ND
−	ND	D	ND	ND	ND	ND	ND	ND	ND	ND	ND
ND	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	ND
ND	ND	−	ND	ND	ND	ND	ND	ND	ND	ND	ND
+	+	d	+	ND	ND	ND	ND	+	+	ND	+
Lys-L-Ala2	ND	Lys-L-Ser2 or Lys-L-Ala-L-Ser	Lys-L-Ala2	Lys-L-Ala-Gly-L-Ala2	Lys-L-Ala-L-Ser	Lys-L-Ser-L-Ala2	Lys-L-Ala-L-Ser	Lys-L-Ala	Lys-L-Ala2 or Lys-L-Ser-L-Ala2	Lys-L-Ala-L-Ser	Lys-L-Ala-(Ser)-L-Ala
Coccoid to elongated cocci	Coccoid to elongated cocci	Coccoid to elongated cocci	Coccoid to elongated cocci	Irregular rods	Small irregular rods	Irregular short coccoid rods with rounded to tapered ends	Irregular short or coccoid rods	Short rods thickened at one end	Coccoid or lenticular	Large spherical or lenticular cells	Short rods
40	37	37–39	38–41.2	39	41–44	44	45	47	37–38	39–40	44–45

livers and kidneys (Hanna et al., 1982), and processed meat products (Borch and Molin, 1988a; Korkeala et al., 1988; Korkeala and Mäkelä 1989; Von Holy and Holzapfel, 1989). Domination of leuconostocs, in addition to meat lactobacilli, was also recorded for dark, firm, and dry (DFD) and normal beef steaks displayed for 3–6 days after wrapping in high oxygen-barrier film (Vanderzant et al., 1983).

In earlier reports, meat-associated leuconostocs were often identified as *L. mesenteroides* subsp. *mesenteroides* or *W. paramesenteroides* (previously *L. paramesenteroides*; Reuter, 1970b; Reuter, 1970d; Collins-Thompson and Rodriguez-Lopez, 1981; Savell et al., 1981; Hitchener et al., 1982; Holzapfel and Gerber, 1986). Their resemblance to acknowledged *Leuconostoc* spp., however, has been doubted (Shaw and Harding, 1984) and in a numerical taxonomic study, Borch and Molin (1988a) found them phenotypically distant from type strains of *Leuconostoc* species. Shaw and Harding (1989) used numerical taxonomy and DNA homology to classify *Leuconostoc* isolates from chill-stored meats and recognized two new species, *L. carnosum* and *L. gelidum*. The taxonomic features of these species are dis-

Table 6. Levels of DNA-DNA relatedness for species of the genera *Leuconostoc* and *Weissella* and *Oenococcus oeni*.

Species or subspecies	% Homology with DNA from:						
	L. carnosum	*L. lactis*	*L. mesenteroides* subsp. *mesenteroides*	*W. confusa*	*W. paramesenteroides*	*W. viridescens*	*L. kimchi*
L. argentinum	ND	35–39	9	ND	ND	ND	7
L. carnosum	78–116	0–25	19–32	ND	0–6	ND	7
L. citreum	ND	23–32	21–39	ND	10–22	ND	2
L. fallax	ND	ND	28–41	ND	25	ND	1
L. gelidum	3–21	0–10	9–31	ND	0–3	ND	17
L. lactis	7–22	74–100	16–49	ND	0–25	ND	2
L. mesenteroides subsp. mesenteroides	1–17	7–38	73–108	ND	7–18	ND	2
L. mesenteroides subsp. cremoris	4–10	5–35	66–106	ND	5–10	ND	24
L. mesenteroides subsp. dextranicum	5–8	6–35	84–110	ND	5–19	ND	2
L. pseudomesenteroides	ND	13–36	18–48	ND	9–22	ND	2
W. confusa	ND	ND	ND	93–105	ND	ND	18–49
W. kandleri						8–9	ND
W. paramesenteroides	0–3	8–14	6–19	ND	82–100	ND	1
W. viridescens	ND	ND	ND	ND	ND	83–92	ND
O. oeni	ND	3–11	8–15	ND	5–7	ND	ND

Abbreviation: ND, not determined.

Data compiled from Dicks et al. (1990), Farrow et al. (1989), Garvie (1976), Hontebeyrie and Gasser (1977), Schillinger et al. (1989), Shaw and Harding (1989) and Vescovo et al. (1979), Kim et al. (2000).

cussed elsewhere. The ability of most strains to grow at 1°C but not at 37°C (Shaw and Harding, 1989) is of practical importance and indicates their typical psychrotrophic character. This is also supported by the data of Savell et al. (1981) and Schillinger and Lücke (Schillinger and Lücke, 1986; discussed in this section), pointing at the competitive behavior of meat leuconostocs at low temperatures of 1–2°C. All strains isolated from processed meat products by Borch and Molin (1988a) grew at 5°C but not at 45°C, and may have similar psychrotrophic character as the Shaw and Harding (1989) strains; their relatedness to the new species, however, is not clear.

Species dominating in vacuum- or modified-atmosphere-packaged meat and meat products include the genera of *Lactobacillus*, *Leuconostoc*, *Carnobacterium*, *Weissella*, *Pediococcus* and *Enterococcus*. With the help of modern taxonomic methods, it has become evident that the major spoilage group previously called "atypical streptobacteria" (Reuter, 1970a; Reuter, 1970b; Reuter, 1970c; Reuter, 1970d; Reuter, 1975) consists of *Lactobacillus sakei* and *Lactobacillus curvatus* (Mäkelä et al., 1992; Vogel et al., 1993; Dykes and von Holy, 1994a; Dykes et al., 1994b; Klein et al., 1996; Torriani et al., 1996). Identification of these LAB to the species level has been the major problem in studies dealing with lactic acid bacterium spoilage of meat. For years *Leuconostoc* species have been associated with meat and meat products, but only the studies employ-

ing modern techniques together with numerical approaches or DNA-based techniques have revealed the occurrence of different *Leuconostoc* species in spoiled meat products. From 1970 to 1989, many studies dealing with leuconostocs associated only *L. mesenteroides* with spoilage of meat and meat products. Later these identification results were considered doubtful (Shaw and Harding, 1984; Borch and Molin, 1988a). The discovery of two psychrotolerant, meat-originated species *L. carnosum* and *L. gelidum* (Shaw and Harding, 1989) clarified the taxonomy of meat-related *Leuconostoc* species.

The successful competition in processed meat systems of *L. amelibiosum* (syn. *L. citreum*), *L. carnosum*, *L. gelidum*, *W. viridescens* and *W. halotolerans*, comparative to typical meat lactobacilli and *Carnobacterium divergens* and *Carnobacterium piscicola*, is explained by their ability to ferment meat carbohydrates competitively under chill conditions and reduced redox potential, and also by their adaptation to the meat substrate (Reuter, 1981; Holzapfel, 1998). While the leuconostocs appear to grow most rapidly on chilled fresh meat (Borch and Agerhem, 1992), *Lactobacillus curvatus* and *Lactobacillus sakei*, on account of their higher tolerance of elevated salt concentrations and nitrite, typically dominate raw fermented sausages, and pasteurized emulsified meat products (Holzapfel and Gerber, 1986; Holzapfel, 1998; Lücke, 1996). Although frequently only an intermediate group,

the role of weissellas and leuconostocs in meat spoilage probably has been underestimated thus far, in relation to the role of the more extensively studied meat-associated lactobacilli (cf. Holzapfel, 1998).

Leuconostoc carnosum and *L. gelidum* clearly play a spoilage role in certain meat products. Both species have been associated with the spoilage of ham products. In a contamination study of vacuum-packaged, cooked, sliced ham (Björkroth et al., 1997; Björkroth et al., 1998), *L. carnosum* was found to be the specific spoilage organism. In this meat plant, *L. carnosum* was found problematic in ham, whereas *Lactobacillus sakei* and *Lactobacillus curvatus* were detected in a variety of products, including many types of sausages. *Leuconostoc gelidum* has been associated with yellow discoloration of ham (Cai et al., 1998b). Thirty-six *L. gelidum* strains were isolated from yellow-pigmented spots occurring on vacuum-packaged ham slices. These isolates possessed 88.8–100% DNA-DNA homology in the reassociation test and 90–95.4% similarity with the *L. gelidum* type strain. They all grew at 4°C and most of the isolates produced yellow pigment also in the reproduction test.

In a study of lactic acid bacteria causing spoilage in vacuum-packaged processed meats, high prevalence of bacteriocin-producing psychrotrophic leuconostocs was revealed (Yang and Ray, 1994). Nine of the isolates were assigned to *L. carnosum*, of which eight originated from different types of ham products and one from sliced turkey product. The *L. carnosum* strains that Shaw and Harding (1989) used for the description of this species were from cold-stored vacuum-packaged beef, pork, bacon, cooked ham and luncheon meat.

The lactic acid bacterium flora on sausages differs from the flora reported to grow on ham. Approximately 36% of the spoilage flora on Vienna sausages was reported to consist of leuconostocs (Dykes et al., 1994c). When these species were identified using DNA-DNA hybridization, the presence of high proportions of *W. paramesenteroides* and the absence of *L. carnosum* was emphasized. *Leuconostoc mesenteroides* subsp. *mesenteroides* and *L. gelidum* were detected in small amounts. Compared to ham and other whole-meat products, emulsion sausages have more variable raw materials and also a different type of processing. Process and ingredients used for ham manufacture may favor the survival and/or growth of *L. carnosum* and *L. gelidum* enabling them to form the dominant spoilage flora.

The spoilage changes caused by the genus *Leuconostoc* follow the same lines as other LAB, particularly the lactobacilli. The most common form of spoilage is formation of sour off-odors and off-tastes but also loosening of the packages due to gas production (Dykes et al., 1994c; Yang and Ray, 1994; Björkroth et al., 2000) has occurred. As heterofermenters, all leuconostocs produce CO_2 from glucose. This may lead to package deformation if gas is produced in excess amounts. Extremely strong and fast gas formation in a modified atmosphere packaged, marinated broiler meat product was described by Björkroth et al. (2000). In this tomato-marinated product, spoilage occurred at 6°C in 4 days and the dominating organism in this spoilage lactic acid bacterium population was *L. gasicomitatum*, which was considered to be a specific spoilage organism in this type of meat product.

Meat and processed, cured meat products apparently provide a favorable growth substrate for some leuconostocs. Refrigeration (2–4°C), in combination with vacuum packaging or reduced (modified) atmospheric conditions, favors the proliferation and eventual domination of leuconostocs and atypical lactobacilli (*Lactobacillus curvatus* and *Lactobacillus sakei*; Reuter, 1981; Holzapfel and Gerber, 1986; Von Holy and Holzapfel, 1989). Pure-culture inoculation of vacuum packaged beef followed by storage at 5°C revealed that growth of a meat-associated leuconostoc was comparable to that of meat lactobacilli, with both strains reaching population levels of >10^8/cm^2 after 7 days (Egan and Shay, 1982). The leuconostocs produced a slightly more severe off-flavor than the lactobacilli. These results were confirmed in part by Schillinger and Lücke (1986) who reported that in vacuum-packaged beef stored at 2°C, the growth of a meat *Leuconostoc* strain used for inoculation was not comparable to that of other LAB. However, in noninoculated meat, *Leuconostoc* strains have very often been found to dominate the population after 4 weeks. It may be concluded that refrigerated storage at 2°C (as compared to 5°C) favors the progressive domination of leuconostocs and typical meat lactobacilli in vacuum-packaged meat (Schillinger and Lücke, 1986). Inoculation studies on lean and fat tissue of beef, pork, and lamb have shown that proliferation of *L. mesenteroides* subsp. *mesenteroides* on lean samples is comparable or slightly better than on fat (Vanderzant et al., 1986). In model studies on the spoilage of commercially marketed meat, Toji (1979) has found the availability of glycogen, other sugars, and protein breakdown products to be key factors determining the growth of leuconostocs and other meat bacteria. Collins-Thompson and Lopez (1982) observed that *Brochothrix thermosphacta* (a typical meat-spoilage bacterium) was able to compete successfully with *L. mesenteroides* subsp. *mesenteroides* in vacuum-packed bologna at 5°C. In a special case of spoilage, refrigerated (1–2°C)

beef strip loins, stored either vacuum packaged or in different combinations of O_2-CO_2-N_2 atmospheres, exhibited a spoilage association that was dominated up to 100% by leuconostocs and was associated with defects such as surface discoloration, general unattractiveness, and off-odors (Savell et al., 1981). For vacuum-packaged beef, domination by *Leuconostoc* spp. representing 45% of the total number of isolates, has been reported by Patterson and Baird (1977). Leuconostocs have also been found as the predominant group, in addition to the *Lactobacillus curvatus*/*Lactobacillus sakei* group, throughout the manufacture and prolonged storage of vacuum-packaged smoked "Vienna-type" sausages (Von Holy and Holzapfel, 1989). Out of 466 representative isolates from spoiled products (with total bacterial numbers of 108–109/g), 36.3% were characterized as *Leuconostoc* spp.; their association with the sausage surface and their relative contribution to the spoilage population was studied by scanning electron microscopy. In addition to souring and slight (though normally not adverse aroma and taste) defects, a leuconostoc-dominated spoilage pattern of vacuum-packaged processed sausages often appears to be accompanied by gas production (blowing) and slime formation. The impact of this spoilage problem is reflected by an average direct loss of 3.5%, based on turnover that can be directly related to lactic acid-bacterial metabolic activities (Von Holy and Holzapfel, 1989).

Fermented, sour aroma and taste were observed as the main sensory defects for vacuum-packed cooked ring sausages (Korkeala et al., 1985), for which leuconostocs were implicated as one of the major microbial groups involved in spoilage (Korkeala and Mäkelä 1989). Korkeala et al. (1988) reported frequent occurrence of ropy slime at the surface of Finnish vacuum-packed cooked meat products, and they found *L. amelibiosum* (syn. *citreum*; Mäkelä et al., 1992) together with *Lactobacillus sakei* responsible for this defect. *Lactobacillus sakei* was the main species associated with this change, and it produced the ropy exopolysaccharide, which in this case is not associated with sucrose as the source for slime formation. Even when the leuconostocs may form slime from sucrose, this capability does not correlate with the formation of ropy slime by *L. carnosum* in meat products (Björkroth et al., 1998).

On the basis of the inhibition of Gram-negative meat spoilage bacteria, leuconostocs were reported to show only moderate potential for ground meat preservation (Dubois et al., 1979). Attempts to utilize leuconostocs in mixed starter cultures for summer sausages produced results inferior of those with *Lactobacillus* cultures alone (Burrowes et al., 1986). Lactic acid bacteria (LAB) have been shown to reduce nitrite in cured meat products, with the highest reduction rate found for *L. mesenteroides* subsp. *mesenteroides* (Collins-Thompson and Rodriguez-Lopez, 1981).

GENUS *WEISSELLA* IN MEAT AND MEAT PRODUCTS *Weissella viridescens* has been associated especially with the greening of meat products (Niven and Evans, 1957). The greening of cured, cooked meat products (Niven et al., 1949; Niven and Evans, 1957; Lörincz and Incze, 1961) is caused by the accumulation of hydrogen peroxide. The H_2O_2 is produced by LAB such as *Weissella viridescens* and *Leuconostoc* spp. under conditions of low E_h. Hydrogen peroxide reacts with nitric oxide hemochromogen and nitric oxide myoglobin in meat. This results in production of greenish oxidized porphyrin (Grant et al., 1988; Holzapfel, 1998). Another problem related to *W. viridescens* in meat processing is the heat resistance of strains contaminating the raw material. It has been demonstrated to survive the regular heat process in sausage manufacture, and is able to survive at 68°C for more than 40 min (Niven et al., 1954; Borch et al., 1988b). These properties may give rise to difficult spoilage problems. The main defects in meat are off-odors and off-flavors, but discoloration and gas production also occur. Together with *Weissella* spp., Borch et al. (1996) also identified *Brochothrix thermosphacta*, *Carnobacterium* spp., *Lactobacillus* spp. and *Leuconostoc* spp. to be associated with the spoilage of refrigerated meat products, causing defects such as sour off-flavors, discoloration, gas production, slime production and decrease in pH. They concluded that a multivariate approach based on spectra of chemical compounds, may be helpful to analyze spoilage, at least for spoilage caused by LAB.

In a study dealing with the LAB from naturally fermented Greek dry salami, *Weissella* species, *Lactobacillus sakei* and *Lactobacillus curvatus* were found to be the dominating organisms (Samelis et al., 1994). From the total of 120 *Weissella* strains, 4 were identified as *W. viridescens*, 11 as *W. hellenica*, another 11 as *W. paramesenteroides*, 31 as *W. minor* and 3 as *W. halotolerans*. The remaining 60 isolates could not be classified under any existing *Weissella* species. These isolates have been considered to represent new unidentified *Weissella* species of meat origin (Tsakalidou et al., 1997). In an attempt to differentiate between *W. viridescens*, *W. paramesenteroides*, *W. hellenica* and some atypical arginine-negative *Weissella* isolates from meat, Samelis et al. (1998) determined their cellular fatty acid composition by a rapid G+C-method.

Dairy Foods

The leuconostocs are more frequently associated with milk and milk products than the weissellas, and may cause undesirable effects in fresh milk. Selected *Leuconostoc* strains, however, are of special value in starter cultures for a variety of fermented milk products, and particularly for imparting a desirable "butter" aroma (diacetyl).

MILK Although leuconostocs, and to a lesser extent, weissellas, are typical contaminants of the dairy environment, they have only limited growth potential in refrigerated milk. Formerly, the leuconostocs were frequently confused with the morphologically similar streptococci (genus *Lactococcus*; Schleifer et al., 1985), which are better adapted to the milk substrate; they can however be clearly distinguished by simple phenotypic criteria such as heterofermentation and production of the D(–)-lactate isomer. Although leuconostocs are regular contaminants of raw and pasteurized milk, relatively little is known about their involvement in and contribution to the spoilage of this product. Earlier reports on the slow development of leuconostocs in refrigerated milk (Juffs and Babel, 1975) were supported by observations of Galli et al. (1983) that *Leuconostoc* spp. (together with lactococci) predominated only when milk was stored at 12°C and not at 5°C. Studies on 182 representative strains of LAB associated with raw milk in Brazil (Antunes and De Oliveira, 1986) showed *L. mesenteroides* subsp. *cremoris* as a minor group, representing only 1.1% of the total. The predominant bacteria involved in the spoilage of pasteurized milk were studied by Keller et al. (1987b), who reported *Leuconostoc* spp. to constitute 9.6% of 258 representative isolates investigated. Using phenotypic criteria, including the cell wall peptidoglycan type, and determination of the mol% G+C in the DNA, Keller et al. (1987b) classified 13 out of 23 strains as *L. mesenteroides* subsp. *mesenteroides*. The remaining strains were represented by *L. lactis* (5), *L. amelibiosum* (syn. *L. citreum*) (4), and *W. paramesenteroides* (1) (Keller, 1987a). Two parameters important in expressing the thermal sensitivity of an organism are D_{10} (decimal reduction time) and Z values. The D_{10} is the time necessary for a 10-fold reduction in viable count at a given temperature. The Z value is the increase in temperature necessary to reduce the D10 by a factor of 10. Decimal reduction (D_{10}) values for these strains, determined at 72°C in full-cream milk, ranged from 4.54 to 5.52. Using different temperatures for determining heat destruction, Z values of 4.39–4.86

were calculated (Keller, 1987a). Generation times of 22 h at 5°C determined in milk (Keller et al., 1987b) indicated a nonpsychrotolerant nature of the strains of *L. mesenteroides* subsp. *mesenteroides* tested, when compared to the criterion of 8 h at 4°C suggested by Law and Mabbit (1983). According to Sadovski et al. (1980) *L. mesenteroides* subsp. *cremoris* showed the slowest growth of starter organisms under psychrotolerant conditions.

In a study of 1760 psychrotrophic bacterial isolates from raw ewe's milk, stored at 4 and 7°C, Nunez et al. (1984) have found predominantly Gram-negative bacteria, yet considered *L. mesenteroides* subsp. *dextranicum* as the main Gram-positive psychrotolerant species. However, no conclusive evidence on the psychrotolerant nature of milk-associated leuconostocs has been provided yet. *Weissella paramesenteroides* was reported to represent a minor group among LAB isolated from salted raw milk, incubated at 30°C for 4–21 d (El-Gendy et al., 1983).

FERMENTED MILK PRODUCTS In contrast to the lactococci, leuconostocs are not competitive growers or important producers of lactic acid in milk. The ability of certain strains to produce the flavor compound diacetyl, however, has led to their frequent incorporation into starter cultures for buttermilk, butter, quarg (cream cheese), and cheese types such as Gouda and Edam (Collins, 1972; Collins and Speckman, 1974; Sandine and Elliker, 1970; Garvie, 1984; Quist et al., 1987). Their use in formulated starter cultures for kefir production has also been reported (Marshall and Cole, 1985; Duitschaever et al., 1987). Cogan et al. (1997) studied 4379 isolates from 35 artisanal dairy products, including 24 artisanal cheeses, and identified 10% of the LAB strains as *Leuconostoc* spp. Lin et al. (1999) have identified *L. mesenteroides* along with *Lactobacillus helveticus* and yeasts (*Kluyveromyces marxianus* and *Pichia fermentans*) as the dominant microorganisms in Taiwanese kefir. *Leuconostoc lactis* and *L. mesenteroides* subsp. *cremoris* contribute to diacetyl production by carbohydrate heterofermentation in associative growth with the lactococci (Garvie, 1984), and their involvement in flavor production even of spontaneously fermented Moroccan dairy products (Iben and Smen) has been documented (Tantaoui-Elaraki and El Marrakchi, 1987). *Weissella paramesenteroides* was found to be the dominant species of LAB in "dadih," a traditional fermented milk in Indonesia (Hosono et al., 1989). Increased flavor production is stimulated at reduced pH, but especially associated with citrate-lyase-positive strains in the presence of

citrate (Speckman and Collins, 1968; Collins and Speckman, 1974; Cogan, 1975). According to Garvie (1984), all strains examined of *L. mesenteroides* subsp. *cremoris* utilized citrate. Walker and Gilliland (1987), however, reported considerable variations in the amount of diacetyl produced among strains of this subspecies. More information on the genetic basis of stability and expression of this feature is needed. The co-metabolism of citrate plus xylose by *L. mesenteroides* subsp. *mesenteroides* resulted in growth stimulation, an increase in D(–)-lactate and acetate production and repression of ethanol production (Schmitt et al., 1997). By contrast, no diacetyl or acetoin was detected in citrate plus glucose fermentation. Zakaria et al. (1998) reported *W. paramesenteroides* as one of three major LAB species in dadih with different strains of *W. paramesenteroides* having different influences on its viscosity and curd syneresis.

In young and hard cheeses, strains of *Lactobacillus* and *Leuconostoc* showed lower counts with longer brining times, in contrast to Micrococcaceae, yeasts and molds. Perez-Elortondo et al. (1998) isolated *L. lactis* as the predominant *Leuconostoc* in Idiazábal ewes' milk cheese.

Representatives of the Genera *Leuconostoc* and *Weissella* Associated with Fish

Reports on the association of leuconostocs and weissellas with fish are rare. Yet, fish and fish products appear to constitute a particular habitat for these groups and especially for the leuconostocs along with other LAB (Ringø and Gatesoupe, 1998).

Together with lactobacilli and carnobacteria, low numbers of leuconostocs were found in association with the spoilage of cold-smoked salmon (Hansen and Huss, 1998), and *L. mesenteroides* subsp. *mesenteroides* and *L. citreum* were reported for spoiled, vacuum-packaged, cold-smoked rainbow trout (Lyhs et al., 1999). Moreover, *L. citreum* was isolated from low-salted fermented fish products (Paludan-Müller et al., 1999). *Weissella thailandensis* was recently described from fermented Thai fish (pla-ra, pla-com; Tanasupawat et al., 2000).

The Genera *Leuconostoc*, *Oenococcus* and *Weissella* in Other Food Habitats

Plant Materials

Vegetables and Fruit Leuconostoc spp. have been reported to predominate on many plant materials (Mundt et al., 1967). However, in addition to lactobacilli, *Weissella* spp. such as *W. viridescens* and *W. confusa*, may also be found occasionally (Kvasnikov et al., 1983; W. H. Holzapfel, unpublished observations). Out of 400 lactic isolates studied by Stirling and Whittenbury (1963), 80% were leuconostocs, 10% pediococci, and the remainder lactobacilli. Among LAB found on plants, *Leuconostoc* appears to be predominant (Mundt et al., 1967). Out of 400 lactic isolates studied by Stirling and Whittenbury (1963), 80% were leuconostocs, 10% pediococci, and the remainder lactobacilli. Species of *Leuconostoc* other than *L. mesenteroides* subsp. *mesenteroides* are only rarely isolated from plant materials (Mundt, 1970). On living undamaged plant tissue, leuconostocs occur in relatively low numbers. They have to compete with less fastidious, mainly Gram-negative bacteria and aerobic sporeformers. The presence of leuconostocs is apparently associated with the release of nutrients from damaged or decaying plant material (Stirling and Whittenbury, 1963). Their numbers increase with the degree of plant maturity (Daeschel et al., 1987) and during harvesting and ensiling. In vegetables such as peas and beans prepared for freezing, *Leuconostoc mesenteroides* subsp. *mesenteroides* may multiply and cause sourness, discoloration, or off-flavor (Sharpe and Pettipher, 1983). *Leuconostoc mesenteroides* subsp. *mesenteroides* dominated among the isolates of LAB responsible for spoilage of fresh ready-to-use grated carrots (Carlin et al., 1989). Villiani et al. (1997) isolated 215 leuconostocs from field grass, natural whey cultures and water-buffalo milk. Of these, 178 were identified as *L. mesenteroides* subsp. *mesenteroides*, while 37 strains could not be identified.

Processed Plant Foods with Low pH Underprocessed or recontaminated juices and beverages containing juice may spoil by formation of slime, CO_2, off-flavors, turbidity, lactic and acetic acid production by acid-tolerant strains of *L. mesenteroides*, and more rarely, also by *W. viridescens* (Back, 1981; Back, 1993).

Fermented Foods of Plant Origin Leuconostoc *mesenteroides* subsp. *mesenteroides* plays an important role in the fermentation of vegetables such as sauerkraut and cucumbers. Although not the dominant species on cabbage at the time of shredding, *L. mesenteroides* subsp. *mesenteroides* initiates the fermentation of sauerkraut and is then succeeded by the more acid-tolerant lactobacilli (Pederson, 1930; Stamer, 1975). At elevated fermentation temperature or increased salt concentration, *Pediococcus cerevisiae* (now named *P. pentosaceus*) may also develop (Pederson and Albury, 1969). The same microbial succession was observed during fermentation of

cucumbers or other pickles as well as olives (Vaughn, 1985). *Leuconostoc mesenteroides* subsp. *mesenteroides* is the first to appear and is quickly succeeded by *Lactobacillus brevis* and *Lactobacillus plantarum*, which then dominate and complete the fermentation. The sequence of LAB is dependent upon the initial load, growth rates, and salt- and acid-tolerances (Daeschel et al., 1987). Kimchi, a traditional Korean food, is produced by the lactic fermentation of vegetables such as Chinese cabbage, radishes and cucumbers. Leuconostocs such as *L. citreum*, *L. gelidum*, *L. kimchi* and *L. mesenteroides* were reported to dominate the early stages of fermentation, followed by lactobacilli (Lee et al., 1997; Kim et al., 2000a; Kim et al., 2000b; Choi et al., 2001), while *Weissella*-like strains were reported for the midstage of fermentation (Choi et al., 2001). *Leuconostoc mesenteroides* subsp. *mesenteroides* is apparently better adapted to plant materials and initiates growth more rapidly than other LAB and produces the acid and carbon dioxide essential for the inhibition of the aerobic mesophilic organisms that might destroy crispness in the cabbage or cucumbers (Steinkraus, 1983). Carbon dioxide produced by leuconostocs replaces the air and creates an anaerobic atmosphere favorable for the stabilization of ascorbic acid and for preservation of the natural flavor of the vegetable (Pederson, 1930). *Leuconostoc mesenteroides* subsp. *mesenteroides* is able to grow at 7.5°C (Vaughn, 1985). *Leuconostoc mesenteroides* subsp. *mesenteroides* is less salt tolerant than the other LAB involved in vegetable fermentation. In salt stock pickles, the initial salt concentration is two- to three-fold higher than that employed in sauerkraut and *L. mesenteroides* subsp. *mesenteroides* therefore plays a less-active role in pickle fermentations (Stamer, 1988). On the other hand, slimy or ropy kraut may be the result of dextran formation caused by *L. mesenteroides* subsp. *mesenteroides* (Vaughn, 1985). *Leuconostoc mesenteroides* subsp. *mesenteroides* has also been found to be involved in the preservation of small-sized ripe tomatoes by lactic acid fermentation (Beltran-Edeza and Hernandez-Sanchez, 1989).

Lavermicocca et al. (1998) reported *L. mesenteroides* subsp. *mesenteroides*, and other *Leuconostoc* spp. as a major group among LAB isolated from table olive leaves, twigs and drupes and from olive brines. Almost all the isolates degraded the glucoside oleuropein but varied in the hydrolysis rate, whereas producers of bacteriocin-like inhibitory substances were only found within the phylloplane population of *L. mesenteroides* subsp. *mesenteroides*, other *Leuconostoc* spp. and *Enterococcus* spp. Most extracellular polysaccharide producers belonged to *L. mesenteroides* subsp. *mesenteroides*.

Leuconostocs have also been found to dominate the early stages during natural fermentation of finger millet (*Eleusine coracana*; Antony and Chandra, 1997). Hancioglu and Karapinar (1997) studied the microflora of Boza, a traditional fermented Turkish beverage, prepared by yeast and lactic acid fermentation of cooked maize, wheat and rice flours. Among the 77 LAB strains isolated during the fermentation, *W. paramesenteroides* (25.6%), *L. mesenteroides* subsp. *mesenteroides* (18.6%), *W. confusa* (7.8%), *L. mesenteroides* subsp. *dextranicum* (7.3%) and *O. oeni* (3.7%) were found. *Weissella confusa* and *W. cibaria* have been associated with Malaysian foods tapai and chili bo (Björkroth et al., 2002). Tapai is sweet, fermented glutinous rice or cassava and chili bo is a nonfermented chili and cornstarch-containing perishable food ingredient.

Leuconostoc mesenteroides subsp. *dextranicum* may be part of the sourdough microbial population and distinctively influences the bread taste (Lönner and Preve-Akesson, 1989). In a phenotypic and molecular study of LAB and yeasts isolated from 25 wheat (*Triticum durum* and *Triticum aestivum*) sourdoughs of Southern Italy, 12% of the isolates were identified as *L. citreum*, and 2% as *Weissella confusa* (Corsetti et al., 2001).

Leuconostoc mesenteroides subsp. *mesenteroides* is also predominant and responsible for initiating the fermentation of many traditional lactic acid fermented foods in the tropics. High numbers of *L. mesenteroides* subsp. *mesenteroides* were isolated from vegetable products like the Indonesian Sayur-Asin prepared from mustard cabbage (Puspito and Fleet, 1985) and from starchy products like cassava (Okafor, 1977) or kocho, an African acidic fermented product from *Ensete ventricos* (Gashe, 1987). Strains of *L. mesenteroides* subsp. *mesenteroides* have been found to produce a highly active linamarase, which hydrolyzes the cyanogenic glucoside linamarin present in cassava (Okafor and Ejiofor, 1985). Gueguen et al. (1997) purified and characterized an intracellular β-glucosidase from a strain of *L. mesenteroides* isolated from cassava. When grown on an arbutin-containing medium, it was found to produce an intracellular β-glucosidase. Its cyanogenic activity was suggested to be of potential interest in cassava detoxification, by hydrolyzing the cyanogenic glucosides present in cassava pulp.

In acidic, leavened breads and similar pancake-like products such as the Indian idli or dosa and the Ethiopian tef (Gashe, 1985), *Leuconostoc mesenteroides* subsp. *mesenteroides* is responsible for souring, strengthening, and leavening the bread (Mukherjee et al., 1965; Steinkraus, 1983; Soni et al., 1986). Using modified Chalmers

medium for differential viable count of mixed starter cultures of LAB in doughs, detected *L. mesenteroides* subsp. *mesenteroides* as part of the mixed microbial populations associated with breadmaking. *Leuconostoc mesenteroides* subsp. *mesenteroides* is also involved in the fermentation of seeds of the African oil bean tree (Antai et al., 1986), and of vanilla beans and cocoa (Ostovar and Keeney, 1973; Passos et al., 1984). *Weissella viridescens* has been reported as a minor group associated with sour dough (Spicher, 1987), and leuconostocs as important fermentation agents of traditional fermented foods from cereals (Holzapfel, 1989; Steinkraus, 1996). Kunene et al. (2000) characterized 180 LAB strains from a sorghum-based fermented weaning food by analysis of soluble proteins and amplified fragment length polymorphism fingerprinting, and found *Lactobacillus plantarum* and *L. mesenteroides* as the dominant species.

Coffee Leuconostoc mesenteroides subsp. *mesenteroides* is also involved in the submerged fermentation of coffee berries, practiced in some highland regions, and by which the oligosaccharide concentration decreases and monosaccharides increase, with a concomitant improvement in coffee quality (Frank and Dela Cruz, 1964; Jones and Jones, 1984; Müller, 1996). In studying the coffee fermentation microflora, Avallone et al. (2001) found the "best" population increase with LAB and yeasts, of which *L. mesenteroides* dominated the LAB population.

Beer O'Sullivan et al. (1999) conducted a comparative study of malthouse and brewhouse microflora. They observed that the microbial count increased dramatically during barley steeping, and although still dominated by pseudomonads, the LAB population significantly increased, with leuconostocs being the predominant LAB detected in the early stages of the process. Vaughan et al. (2001) studied bacteriocins produced by LAB isolated from malted barley. Among the eight strains of LAB isolated from various types of malted barley, four *L. mesenteroides* strains were shown to produce bacteriocins highly similar or identical to leucocin A, leucocin C or mesenterocin Y10[5], all typical of Class II bacteriocins. Although less frequently than pediococci, *L. mesenteroides* may be associated with the spoilage of beers with low hops content (Donhauser, 1993). In addition, *L. mesenteroides* and *W. paramesenteroides* may occasionally be involved in the spoilage of beers with pH values >4.8 and of low alcohol and alcohol-free beers, ales, and beer mixes (coolers and shandys; Back, 1994).

Apple Cider Laplace et al. (1998) studied the incidence of indigenous microflora associated with the environment of traditional French cider making. In the traditional cider, among the LAB, *O. oeni* dominated, and malolactic conversion was observed. By contrast, no development of LAB was observed in a second cider not produced under conventional procedures.

Wine Some leuconostocs, lactobacilli and pediococci are associated with the early stages of fermenting grape must (juice). *Oenococcus oeni*, however, has been reported as the most important and desirable species among the LAB involved in wine making thanks to its key role in the secondary fermentation of wine, also referred to as the "malolactic fermentation" (MLF). By their high resistance to SO_2 and ethanol, the oenococci may be present in relatively high numbers at the end of the alcoholic fermentation. At this stage they play the major role in the production of microbiologically stable wines by converting L-malic acid to L(+)-lactic acid; this also causes a rise in the wine pH by 0.1–0.3 units (Davis et al., 1985a; Wibowo et al., 1985). The MLF is considered as important for improving the sensory properties of wines (Rossi et al., 1978; Rossi et al., 1993; Rodriguez et al., 1990); this is especially important in wine regions with moderate to cool climates. However, not all strains of *O. oeni* appear equally suitable for the MLF, and Edwards et al. (1998) identified two strains that were associated with sluggish and/or stuck fermentations, and that were found to slow down some alcoholic fermentations. Moreover, high pH wines are, however, more susceptible to spoilage by different LAB. *Leuconostoc mesenteroides* subsp. *mesenteroides* and *L. mesenteroides* subsp. *dextranicus*, and some strains of *O. oeni*, may be the causative organisms of slime formation in wine, while some leuconostocs may also be responsible for mannitol off-taste in wine (Dittrich, 1993). Coton et al. (1998a) studied histamine-producing LAB in wines, and observed that around 50% of the *O. oeni* strains contained the histidine decarboxylase gene, and were the major group responsible for production of histamine in wine.

Silage In silage, LAB are responsible for the primary acid fermentation, which is essential to prevent multiplication of clostridia (Whittenbury et al., 1967). Fermentation is usually initiated by *Enterococcus faecalis* and *L. mesenteroides* subsp. *mesenteroides*, which replace the aerobic Gram-negative microbial population initially present on herbage intended for silage (Daeschel et al., 1987). Eventually leuconostocs are in turn superseded by aciduric

Table 7. Selective media for lactobacilli and related genera (LLPW-group).

Medium	Application	Groups or species to be cultivated	Inhibitor/indicator	References
Rogosa (pH 5.5)	General	Lactobacilli, *Weissella, Leuconostoc* and *Pediococcus*	Acetate, and acetic acid	Rogosa et al., 1951
NAP (pH 5.5)	Meat	Lactobacilli, lactococci, and (*Leuconostoc* ?)	Nitrite, actidione, and polymyxin B	Davidson and Cronin, 1973
MRS (pH 5.5)	General, meats	Lactobacilli and *Weissella*	None	Baird and Patterson, 1980
MRS-S (pH 5.7)	General, meats	Lactobacilli, *Leuconostoc,* and *Weissella*	Sorbic acid (0.1%)	
LaS (pH 5.0)	Meats	Lactobacilli, (*Leuconostoc,* and *Weissella*)	Sorbic acid (0.04%)	Reuter, 1970a
HHD (pH 7.0)	Fermented vegetables	Homo- and heterofermentative LAB	Bromocresol green	McDonald et al., 1987
M 5 (pH 6.5)	Wine	Homo- and heterofermentative LAB	Bromocresol green	Zúñiga et al., 1993
TJA (pH 5.0)	Wine	Lactobacilli, pediococci, and *Leuconostoc*	Cycloheximide, sorbic acid	Yoshizumi, 1975

Abbreviations: NAP, nitrite-actidione-polymyxin medium; MRS, DeMan Rogosa Sharpe medium; LaS, *Lactobacillus*-sorbate medium; HHD, homofermentative-heterofermentative differential medium; and TJA, tomato juice agar.
Modified from Schillinger and Holzapfel (2002).

lactobacilli (Langston et al., 1962; Woolford, 1985). Investigating the microbial population of high-moisture corn grain, Dellaglio et al. (1984) have found that at ensiling, 67% of the LAB consisted of leuconostocs and heterofermentative lactobacilli and that after 120 days, 84% of the isolates belonged to the homofermentative lactobacilli. Leuconostocs isolated by Dellaglio and Torriani (1986) and Dellaglio et al. (1984) from ensiled high-moisture corn grain and maize (corn) silage were identified as *W. paramesenteroides* by using DNA-DNA hybridization techniques. Grazia and Suzzi (1984) report the occasional occurrence of leuconostocs in Italian maize and alfalfa silage.

Sugar Leuconostocs are known to be responsible for deterioration effects in the sugar industry, and are one of the first bacterial groups studied for their causative role in commercial losses (Van Tieghem, 1878). During harvesting, sugar cane is contaminated with *L. mesenteroides* subsp. *mesenteroides*, which is able to grow within the cut stalks and cause souring of the cane juice (Tilbury, 1975). The high sugar content (about 15%) and the initial pH of 5.0–5.5 render cane juice a suitable substrate for LAB. In hot, humid climates, growth of *L. mesenteroides* subsp. *mesenteroides* in harvested cane may result in a loss of 1–5% of total sugar for each day between harvesting and processing (Tilbury, 1975). More-

over, large amounts of dextran may be synthesized from sucrose by *L. mesenteroides* subsp. *mesenteroides* causing undesirable complication of the refining process (Pivnick, 1980). In cane sugar plants, similar spoilage is caused by slime-producing strains of *L. mesenteroides* subsp. *mesenteroides* and sugar-tolerant (15% sucrose), acidophilic strains of *W. confusa*, also causing souring (Sharpe et al., 1972). The ability of *L. mesenteroides* subsp. *mesenteroides* to produce dextrans from sucrose by a dextransucrase has been exploited for the production of commercially valuable dextrans on an industrial scale and is mentioned elsewhere in this chapter.

Carrots Forty *Leuconostoc* strains from commercial ready-to-use shredded carrots were characterized by physiological and genetic tests (Torriani et al., 1999). Most of them were identified by RAPD-PCR as *L. mesenteroides* subsp. *mesenteroides*, two strains as *Leuconostoc citreum*, and one as *Leuconostoc pseudomesenteroides*. Using in vitro antagonistic assays, it was demonstrated that *Leuconostoc* isolates were inhibited by selected strains of facultatively heterofermentative lactobacilli able to produce antimicrobials; *Lactobacillus plantarum* IMPC LP4 showed the strongest inhibitory activity. Its application as inoculant in shredded carrots was effective in controlling the growth of leuconostocs, which were the possible cause of deteriora-

tion during storage. This hurdle could be readily combined with low temperatures to reduce microbial spoilage risk of ready-to-use carrots.

Soy Products A strain of *L. mesenteroides* subsp. *mesenteroides*, found to spoil Juten-tofu by formation of "yellow spots," was isolated on a soy milk agar plate at 5°C under anaerobic conditions (Matsuzawa et al., 1998). Contamination probably occurred after heating of a package during Juten-tofu manufacturing.

Isolation

Members of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Weissella* (so-called "LLPW group"), share a number of physiological similarities and generally respond in the same way to conditions or compounds inhibitory to non-lactic acid bacteria (non-LAB). Therefore, most culture media developed for the detection of *Lactobacillus*, *Weissella* or *Leuconostoc* are not completely selective for the particular genus; this principle is illustrated by the media and their applications, shown in Table 7. However, carnobacteria can easily be distinguished from the LLPW group by their non-aciduric nature, and their ability to grow at pH values up to 9.5, a property that is also shared with most enterococci. Knowledge of the physiology and typical

environmental conditions favoring growth of specific strains are important prerequisites for the successful isolation of *Leuconostoc* and *Weissella* species. Both genera are typically associated with mixed populations of LAB in natural and artificial plant and food environments. The complex growth requirements and general physiological features of most leuconostocs and weissellas are comparable to those of the lactobacilli, pediococci, and other LAB. These aspects explain the difficulty of obtaining pure *Leuconostoc* and/or *Weissella* cultures in a one-step selective operation.

Information on the composition of general media for the cultivation, maintenance, and semiselective isolation of leuconostocs still has actual importance as originally compiled by Garvie (1984), and is summarized in Table 8. Generally, these media also support growth and enable selective and semiselective cultivation of weissellas.

Isolation from Plant Material

Plant materials such as grass, herbages, and vegetables are the natural habitat of several types of LAB. Ensilage allows the enrichment of most *Leuconostoc* spp. in addition to pediococci and lactobacilli. Favorable conditions for the initial dominance by leuconostocs exist in spontaneous vegetable fermentation processes, involving 2%

Table 8. Ingredients of the various media used in the identification of species of the genera *Leuconostoc*[a] and *Weissella*.

Ingredient	Medium[b]						
	YGPB	MRS	Medium 1	Medium 2	ATB	CMB	DTB
Glucose	1.0	2.0	1.0	1.0	1.0	1.0	1.0
Peptone	1.0	1.0	1.0	1.0	1.0	1.0	0.75
Meat extract	0.8	0.8					
Yeast extract	0.3	0.5	0.5	0.5	0.5	0.5	0.25
NaCl	0.5						
KH_2PO_4	0.25		0.5	0.5		0.25	0.25
K_2HPO_4	0.25	0.2					
$MgSO_4 \cdot 7H_2O$	0.2	0.2	0.2	0.2	0.2	0.2	0.2
$MnSO_4 \cdot 4H_2O$	0.005	0.005	0.005	0.005	0.005	0.005	0.005
Ammonium citrate		0.2	0.5				0.1
Citric acid				0.5		0.25	
DL-Malic acid						0.25	
Sodium acetate		0.5	0.25	0.25			0.25
Tween 80		0.1	0.1	0.1		0.1	0.1
Tomato juice				10.0	25.0		10.0
Usual pH	6.8	6.2	6.5	4.8	4.8	4.8	6.5

[a]Values are given in percent (w/v) of each ingredient. Totally, 1.5% agar is added when solid media are required, and 0.05% cysteine hydrochloride is added to all media when required. Sterilization is normally at 15 lb for 15 min. Special techniques used with different media are mentioned in the text.
[b]YGPB, yeast glucose phosphate broth (Garvie, 1976); MRS, (DeMann et al., 1960); media 1 and 2: (Garvie, 1969); ATB, acid tomato broth (Garvie and Mabbitt, 1967); CMB, citrate-malate broth (Garvie and Mabbitt, 1967); and DTB, dilute tomato broth (Garvie and Mabbitt, 1967).
From Garvie (1984).

brine. In these environments, leuconostocs are selectively enriched during the early stages of fermentation. The choice of semi- or nonselective media, such as De Man Rogosa Sharpe (MRS) medium (De Man et al., 1960) or APT (Evans and Niven, 1951) for isolation often depends on conditions where leuconostocs predominate.

Ensilage Enrichment of Lactococci, Streptococci, Leuconostocs, and Pediococci

The method is as described by Whittenbury (1965b). Grasses and other plant material are collected and cut in pieces as aseptically as possible. The prepared material is then placed into sterile glass tubes and compressed. Fifty grams of material are enough to fill a 3×20 cm tube. The tubes are sealed in a way that permits gas under pressure to escape but prevents the entry of oxygen. A number of silages is prepared and incubated at 30°C. Tubes are opened beginning on the second day. The silage is removed and placed into flasks containing sterile water, which are vigorously shaken. Then, these suspensions can either be spread directly onto agar plates or diluted and pour-plated. The 2- to 3-day-old silages are the best sources for lactococci, streptococci and leuconostocs.

A modification of the above method was reported by Weiler and Radler (1970). Grape leaves are homogenized with the same amount of acetate buffer (pH 5.4; 0.2 M). The homogenate is placed into sterile tubes. The tubes are sealed and incubated at 30°C.

Tetrazolium-sucrose (TS) Agar for Isolation of Leuconostocs and Streptococci (Mainly Lactococci) from Thawed Frozen Peas and Vegetables (Cavett et al., 1965)

Evans peptone	10 g
Lab-lemco	10 g
Sucrose	50 g
New Zealand agar	14 g
Distilled water	1 liter

Adjust to pH 6.0. Lots of 100-ml aliquots are sterilized at 121°C for 20 min, and 1 ml of a 1% solution (w/v) of filter-sterilized 2,3,5-triphenyltetrazolium chloride (TTC) is added per aliquot. The leuconostocs produce translucent, glassy, or watery colonies, containing gummy polysaccharide.

Thallous-acetate-tetrazolium-sucrose (TTS) Agar for Isolation of Leuconostocs from Plant Materials (Cavett et al., 1965)

Satisfactory results of leuconostoc isolation were obtained by modification of the Barnes (1956) medium. Glucose was substituted by sucrose, and 1 ml of filter-sterilized 10% solution of thallous acetate was added to 100 ml of the TS medium (Cavett et al., 1965).

Differential Medium for the Enumeration of Homofermentative and Heterofermentative LAB from Fermented Vegetables (HHD-medium; McDonald et al., 1987)

Fructose	2.5 g
KH_2PO_4	2.5 g
Trypticase peptone	10.0 g
Phytone peptone	1.5 g
Casamino acids	3.0 g
Yeast extract	1.0 g
Tween 80	1.0 g
Bromocresol green	20.0 g
Agar (if applicable)	20.0 g

Dissolve the medium components in distilled water and q.s. up to 1 liter. The pH of the dissolved medium is adjusted to 7.0 ± 0.02 and the medium is autoclaved at 121°C for 15 min. The bromocresol green is prepared as a stock solution by dissolving 0.1 g of bromocresol green in 30 ml of 0.01 N NaOH. On the agar medium, homofermentative colonies are blue to green, while heterofermentative colonies remain white (McDonald et al., 1987).

Glucose-Yeast Extract Agar for Isolation of *Leuconostoc* and *Pediococcus* (Whittenbury, 1965b)

Glucose	5.0 g
Yeast extract	5.0 g
Peptone	5.0 g
Meat extract	5.0 g
Agar	15 g

Dissolve the medium components in distilled water and q.s. up to 1 liter and adjust pH to 6.5.

Acetate Agar for Isolation of *Leuconostoc* and *Pediococcus* (Whittenbury, 1965a)

Meat extract	50 g
Peptone	5.0 g
Yeast extract	5.0 g
Glucose	10 g
Tween 80	0.5 ml
Tap water	900 ml

The pH is adjusted to 5.4. Medium is autoclaved at 121°C for 15 min. Before plating, 100 ml of sterile 2 M acetic acid-sodium acetate buffer (pH 5.4) is added. This agar is a modification of the medium proposed by Keddie (1951) as being selective for lactobacilli.

Yeast Extract-Glucose-Citrate (YGC) Broth for Isolation of *Leuconostoc* (Garvie, 1967a)

Peptone	10.0 g
Lemco	10.0 g
Yeastrel (yeast extract)	5.0 g
Glucose	10.0 g
Triammonium citrate	5.0 g
Sodium acetate	2.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$MnSO_4 \cdot 4H_2O$	0.05 g
Tween 80	0.05 ml

Dissolve the medium components in distilled water and q.s. up to 1 liter and adjust the pH to 6.7. The medium is autoclaved at 121°C for 15 min. YGC has been suggested for the isolation and cultivation of leuconostocs from different sources.

Medium for Isolation of *L. mesenteroides* subsp. *mesenteroides* from Plant Materials (Vrbaski et al., 1988)

Sucrose	100.0 g
Yeast extract	2.5 g
K_2HPO_4	5.0 g
Ammonium sulfate	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.6 g
Agar (if necessary)	20.0 g

Dissolve the medium components in distilled water and q.s. up to 1 liter. The pH is adjusted to 7.8 before sterilization at 114°C for 20 min. Growth of *L. mesenteroides* subsp. *mesenteroides* results in a highly viscous broth medium within 24 h (Vrbaski et al., 1988).

Isolation from Meat and Meat Products

MRS-agar, adjusted to pH 5.7 and with 0.2% of potassium sorbate added, allows the selective isolation of leuconostocs and lactobacilli from meat products. No direct differentiation between lactobacilli and leuconostocs is possible on this medium. Reuter (Reuter, 1970e; Reuter, 1985) recommended the use of thallium acetate medium according to Barnes (1956) for the selective isolation of leuconostocs from meat in the presence of a predominating lactobacilli population. Carnobacteria have been found resistant to thallium acetate, and may grow on this medium, especially if adjusted to pH >7.0 (W.H. Holzapfel, unpublished observations). Elective and selective media that may find application in the isolation and detection of *Leuconostoc* and *Weissella* spp. and other LAB have been discussed by Holzapfel (1998) and are summarized in Table 7.

Isolation from Milk and Dairy Products

Most semiselective media for leuconostocs also support growth of lactobacilli and pediococci and do not allow differentiation between these groups. These include media such as MRS (De Man et al., 1960) or Rogosa SL-medium (Rogosa et al., 1951); colonies on these media need to be further identified. General aspects concerning these and related media for lactobacilli and pediococci are discussed elsewhere.

To control gas and aroma (diacetyl) production in the fermentation of various dairy products, it is important to know the quantitative composition of the starter cultures used. *Leuconostoc* species and *Lactococcus lactis* subsp. *diacetylactis* are components of many mesophilic starter cultures. These organisms are able to ferment citrate with concomitant production of CO_2 and diacetyl.

For the collective enumeration of leuconostocs and *Lactococcus lactis* subsp. *diacetylactis* in

starters and fermented dairy products, a whey agar containing calcium lactate and casamino acids (WACCA) has been introduced by Galesloot et al. (1961).

WACCA 0.5% Medium for Enumeration of *Leuconostoc* and *Lactococcus lactis* subsp. *diacetylactis* (Galesloot et al., 1961).

Dissolve 5 g of Ca-lactate $\cdot 5H_2O$, 7 g of casitone, and 0.5% yeast extract in 1 liter of whey. Adjust to pH 7.3 with $Ca(OH)_2$-suspension. Steam for 30 min. Filter and adjust to pH 7.1 with a NaOH solution. Add 1 ml of $MnSO_4$ solution (40 mg $MnSO_4 \cdot 4H_2O$/100 ml). Dissolve 15 g of agar. Clarify with 5 g of albumin. Sterilize for 15 min at 110°C (15 ml/tube).

Preparation of whey:

Add 0.3 ml of 35% $CaCl_2$ solution and 0.3 ml of commercial rennet (strength 1:10,800) to 1 liter of high-temperature-short-time (HTST)-pasteurized fresh skim milk at 30°C. Cut coagulum after 30 min at 30°C. Filter after 2 h at 45°C.

Preparation of Ca-citrate suspension:

Suspend 28 g of Ca-citrate (Merck) in a 100 ml of 1.5% carboxymethylcellulose solution, prepared at 45°C. Allow to precipitate for 2 h at 45°C. The supernatant is steamed for 30 min.

Application:

Add 0.3–0.7 ml of Ca-citrate suspension per 15 ml of WACCA 0.5% (48°C). The amount to be added has to be adapted to the type of starter culture under investigation. Incubate for 5 days at 25°C. Count after 2, 3 and 5 days.

A different medium (KCA) developed by Nickels and Leesment (1964) for the same purpose yields comparable results.

For the selective isolation and enumeration of *Leuconostoc* strains from mixed strain starter cultures, the following medium has been proposed.

HP Medium for Enumeration of Leuconostoc (Pearce and Halligan, 1978)

Phytone	20.0 g
Yeast extract	6.0 g
Beef extract	10.0 g
Tween 80	0.5 g
Ammonium citrate	5.0 g
$FeSO_4 \cdot 7H_2O$	0.04 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$MnSO_4 \cdot 4H_2O$	0.05 g
Glucose (filter sterilized separately)	10.0 g

Dissolve the medium components in distilled water and q.s. up to 1 liter. The addition of tetracycline (0.12 µg/ml) to the medium selectively inhibits the growth of most streptococci, and, therefore, allows the direct enumeration of *Leuconostoc* species in mixed-strain cultures.

A rapid enzymatic method has been found suitable for the quantitative enumeration of *Lactococcus lactis* in mixed cultures (Boquien et al., 1989). This method relies on the linear correla-

tion that was shown to exist between population numbers of *Lactococcus. lactis* (strain CNRZ 1091) and the activities of α-galactosidase and citrate lyase. It enables the estimation of a bacterial population within 2 h (Boquien et al., 1989). The method may have limited application for undefined mixed populations since some LAB other than leuconostocs may produce similar enzymes.

Isolation from Wine

The former "wine" leuconostocs (now *Oenococcus oeni*) differ in a number of physiological properties from the leuconostocs. Media for their selective enumeration rely on their acidophilic nature (Garvie and Farrow, 1980b), alcohol tolerance, and adaption to the wine environment. Several acidic media have been employed for the isolation of *O. oeni* and of pediococci from wine (Garvie, 1967a; Weiler and Radler, 1970).

Acidic Tomato Broth (ATB) for Isolation of *Leuconostoc* and *Pediococcus* (Garvie, 1967a)

Peptone	10.0 g
Yeastrel	5.0 g
Glucose	10.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$MnSO_4 \cdot 4H_2O$	0.05 g
Tomato juice	25% (v/v)

Dissolve the medium components in distilled water and q.s. up to 1 liter. The pH is 4.8 and the medium is autoclaved at 121°C for 15 min. Before use, a solution of cysteine hydrochloride sterilized by filtration is added to a final concentration of 0.05% (w/v).

Isolation of Lactic Acid Bacteria from Wine (Weiler and Radler, 1970)

Peptone	5.0 g
Yeast extract	5.0 g
Glucose	10.0 g
Diammonium hydrogencitrate	2.0 g
Sodium acetate ·3H$_2$O	5.0 g
Tween 80	1.0 g
KH$_2$PO$_4$	5.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$MnSO_4 \cdot 4H_2O$	0.2 g
$FeSO_4 \cdot 7H_2O$	0.05 g
Agar	15.0 g

Dissolve the medium components in distilled water and q.s. up to 1 liter. The pH is 5.3–5.4. Medium is autoclaved 15 min at 121°C. To inhibit growth of yeasts, the medium is supplemented with sorbic acid at a final concentration of 0.05%. This medium is not completely selective for *O. oeni*.

Davis et al. (1985a) recommended the use of either MRS agar (De Man et al., 1960) or tomato juice agar (Ingraham et al., 1960), adjusted to pH 5.5 and supplemented with 50 μg of cycloheximide per ml for the direct isolation of *O. oeni* from wine. Supplementation of the general medium

with 40–80% wine, has been found to enhance growth of *O. oeni* (Davis et al., 1985b).

Fructose and Tween 80 (FT)-Medium for *O. oeni* (Cavin et al., 1988)

Casamino acids	5 g
Yeast extract	4 g
KH$_2$PO$_4$	0.6 g
KCl	0.45 g
CaCl$_2$ ·2H$_2$O	0.13 g
$MgSO_4 \cdot 7H_2O$	0.13 g
$MnSO_4 \cdot H_2O$	0.003 g
Tween 80	1 ml
L(–)-Malic acid	10 g
Agar (as desired)	15 g
D(+)-Fructose	35 g
D(+)-Glucose	5.0 g

Dissolve the medium components in distilled water and q.s. up to 1 liter. The pH of the medium is adjusted with 10 N NaOH to 5.2, and then autoclaved at 121°C for 15 min.

Malo-lactic Differential (MLD) Medium for Screening *O. oeni* Strains Defective in Malolactic Fermentation (Cavin et al., 1989)

This medium is based on the FT medium (discussed previously; Cavin et al., 1988), which is supplemented with 100 g of cellulose MN 300 and 0.1 g of bromocresol green per liter. Lower concentrations of fructose (4 g/liter) and glucose (1 g/liter) substitute for those used in the FT medium, and are added to the sterilized medium as filter-sterilized stock solutions consisting of 8% fructose and 2% glucose (Cavin et al., 1989). Colonies from organisms defective in malolactic fermentation, show an acid reaction and remain yellow-green, while the malolactic positive colonies turn blue.

Dicks (1989) Recommended Acidic Grape Broth for Cultivation and Maintenance of *O. oeni* Strains

Glucose	10 g
Peptone	10 g
Yeast-extract	5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$MnSO_4 \cdot H_2O$	0.05 g
Grape juice	250 ml

Dissolve the medium components in distilled water and q.s. up to 1 liter. The pH is adjusted to 4.8 with 10 N NaOH.

The reader is also referred to procedures used by Nonomura et al. (Nonomura et al., 1965; Nonomura et al., 1967) for studying malolactic bacteria from wine. Summarizing information is given in Table 9.

Media for Physiological Studies

Basal MRS-media

MRS-broth without citrate, acetate, beef extract, or glucose (pH 6.5) and with 0.004% chlorophenol red indicator added, can be used

Table 9. Summary of media that may be used for the isolation of *Oenococcus oeni* from wine.

Medium	pH	Selectivity	Most important components	References
Acidic tomato broth (ATB)	4.8	Semi-selective	Tomato juice (25%)	Garvie, 1967
Weiler and Radler medium	5.3–5.4	Semi-selective	Sodium acetate (0.5%), Tween 80 (0.1%), and sorbic acid (0.05%)	Weiler and Radler, 1970
M104 medium	4.5	Selective	Tomato juice (25%), DL-malic acid (1%), and Tween 80 (0.1%)	Barillière, 1981
Tomato juice-glucose-fructose-malate broth (TGFMB)	5.5	Selective	Tomato juice (diluted), fructose (0.3%), malate (0.2%), Tween 80, and cycloheximide (0.005%)	Izuagbe et al., 1985
Fructose and Tween 80 (FT 80) medium	5.2	Semi-selective	Fructose (3.5%), L-malic acid (1%), and Tween 80 (0.1%)	Cavin et al., 1988
Enriched tomato juice broth (ETJB)	4.8	Semi-selective	Tomato juice broth (Difco) (2%), Tween 80 (0.1%), and cysteine (1%)	Britz and Tracey, 1990
Universal beer agar (UBA)		Selective	Apple juice (20%) and cycloheximide (0.005%)	Rodriguez et al., 1990

From Schillinger and Holzapfel (2002).

for determination of the sugar fermentation pattern. In another modification, MRS-broth without acetate or citrate (BM-MRS-broth) was recommended (Wilkinson and Jones, 1977; Shaw and Harding, 1989).

Sugar Basal Broth (Garvie, 1984) for Sugar Fermentation

Oxoid peptone	10.0 g
Yeast extract	2.5 g
Tween 80	0.1 g
Bromocresol purple (1.6% solution in ethanol)	1.0 ml

Dissolve the medium components in distilled water and q.s. up to 1 liter. Adjust pH to 6.8. Add 5-ml amounts to test tubes, and autoclave at 121°C for 15 min.

Sugar Basal Broth for Sugar Fermentation Pattern of *O. oeni* (Garvie, 1984).

The normal "sugar basal broth" mentioned before can be modified for *O. oeni* by adjusting the pH to 5.2, including 0.5% agar, and substituting 0.004% bromocresol green for bromocresol purple (Garvie, 1984).

MRS-broth Without Citrate (Holzapfel and Gerber, 1983).

To study gas production from glucose, growth on MRS-broth without citrate, using Durham tubes (Holzapfel and Gerber, 1983) is recommended. Gibson's medium (Gibson and Abdel-Malek, 1945) may give better results for slow gas producers (Briggs, 1953), and was recommended by Borch and Molin (1988a).

Milk Agar (Garvie, 1984)

Litmus milk	800 ml
Glucose	5.5 g
Yeast extract	2.0 g
Tomato juice (Oxoid)	100 ml
Nutrient agar	200 ml

Mix all ingredients except the agar, and adjust to pH 6.8; heat to 45°C and add the agar already melted. Add 10-ml aliquots to vials, and autoclave at 115°C for 10 min. Cool in water. For use, melt, cool to 45°C, and inoculate with 0.25 ml of culture. Pour onto Petri dishes, allow to solidify, and pour 4.0 ml of nutrient agar as top layer (Garvie, 1984).

Modified MRS Agar for Dextran Production.

To study dextan production, MRS-agar modified by the addition of 10% (w/v) sucrose (Sharpe, 1962) is one of two recommended media. The second one follows.

Sucrose Agar for Dextran Production (Garvie, 1984)

Tryptone	10.0 g
Yeast extract	5.0 g
K_2HPO_4	5.0 g
Diammonium citrate	5.0 g
Sucrose	50.0 g
Agar	15.0 g

Dissolve the medium components in distilled water and q.s. up to 1 liter. Adjust pH to 7.0 and autoclave at 121°C for 15 min.

Chemically Defined Medium for the Growth of *L. mesenteroides*.

A chemically defined medium for the growth of *L. mesenteroides* was developed by Foucaud et al. (1997). The medium contained lactose, Mn^{2+}, Mg^{2+}, 12 amino acids, eight vitamins, adenine, uracil and Tween 80. A beneficial effect on growth was shown for aerobic conditions and also for potassium phosphate (135 mM) as a suitable buffer. A growth rate of 0.85 ± 0.10 h^{-1} was determined for the six strains examined, while cell densities up to 3.5×10^9 colony forming units (CFU)/ml were reached.

General Growth Conditions

Most leuconostocs are relatively insensitive to oxygen, although more prolific growth is often observed under reduced atmospheres. Especially for the isolation of leuconostocs from vacuum-packaged meats, and for *O. oeni* from wine, microaerophilic to anaerobic conditions are recommended. Gas mixtures of N_2, H_2, or CO_2 or anaerobic "gas-generating kits," such as GasPak (Oxoid) or Anaerocult A (Merck), provide more favorable conditions for surface colony growth. Cysteine hydrochloride (0.05–0.1%) may be added to broth media. Incubation temperatures should be 20–25°C with the lower range being more favorable for *O. oeni*. Depending on the strain and other growth factors, an incubation period from 48 h up to 10 days may be necessary.

Identification

Leuconostoc species and *O. oeni* can be differentiated from the other LAB by the following criteria: ovoid appearance, formation of gas from glucose, inability to hydrolyze arginine, and by the production of the D(–)-lactate isomer from glucose. These properties are also typical of the ovoid-shaped weissellas. The ability to produce gas and D(–)-lactic acid from glucose are the phenotypic key factors distinguishing leuconostocs and ovoid-shaped weissellas from lactococci, streptococci, enterococci and homofermentative coccoid lactobacilli. Heterofermentative lactobacilli produce the DL-lactate isomer and the shape of the cell is more rod-like.

The differentiation of the genus *Weissella*, as a whole, from the other LAB is, however, more problematic. This genus contains organisms producing either D(–)- or DL-lactate, and some species are able to hydrolyze arginine. The morphology of the species in the genus *Weissella* varies from ovoid to rod-like. Therefore, they may be mistaken for heterofermentative *Lactobacillus* species. Reliable phenotypic identification of weissellas may demand special methods, such as analysis of the peptidoglycan interpeptide bridge, DNA-DNA relatedness, or numerical approaches.

In Table 1, a number of key features of the genera *Leuconostoc* and *Weissella* is summarized in comparison with those of other genera of the LAB. These data illustrate the difficulty of identifying *Weissella* strains on the basis of phenotypic characteristics.

Phenotypic Species Differentiation

Differentiation of leuconostocs and weissellas by phenotypic means is sometimes problematic.

Table 5 shows the phenotypic characteristics of *Leuconostoc* spp., *Weissella* spp. and *O. oeni*. *Oenococcus oeni* can be recognized easier owing to its ability to grow in the presence of ethanol (10%) and in/on acidic media. It tolerates pH 4.2–4.8, and growth has occurred even at lower pH values. *Leuconostoc* species are generally not that acid-tolerant.

Leuconostoc sensu stricto species are difficult, sometimes impossible, to distinguish by phenotypic routine testing. Many reactions are strain-dependent or are, on the other hand, shared between the different species. On the basis of the characterization results of 81 *Leuconostoc* strains (Milliere et al., 1989), it was concluded that routine phenotyping does not enable good species identification. Reliable differentiation of *L. carnosum* from *L. gelidum* has been stated to demand DNA-DNA reassociation (Dellaglio et al., 1995).

Leuconostoc citreum may produce yellow pigment, which can aid in its identification. Formation of dextran from sucrose has been used as one criterion differentiating *Leuconostoc* species. The value of this test may, however, be lowered when more information on the phenotypic reactions is gained from well-characterized *Leuconostoc* populations. Björkroth et al. (1998) reported that dextran production was associated with 11 from the total of 25 *L. carnosum* bacterial types distinguished by pulsed-field gel electrophoresis. This characteristic had previously been considered common for all *L. carnosum* strains.

Differentiation of *Weissella* from *Leuconostoc* species may be challenging. *Weissella confusa*, *W. halotolerans*, *W. kandleri* and *W. minor* can be distinguished by their ability to hydrolyze arginine, produce DL-lactate from glucose, and by their rod-shaped morphology. However, identification of *W. paramesenteroides*, *W. hellenica* and *W. viridescens* and distinguishing them from leuconostocs, demands the use of several biochemical tests. The interpeptide bridge of the peptidoglycan may particularly serve as a strong phenotypic feature for this group.

Carbohydrate Fermentation Patterns

Carbohydrate fermentation patterns alone are of limited use in the identification of *Leuconostoc sensu stricto* species because of the considerable variation between strains. Milliere et al. (1989) stated that only the attribution of a taxonomic weight to some carbohydrate fermentation tests and the use of genetic analyses can resolve strain identification within *Leuconostoc* species. Only *L. mesenteroides* subsp. *cremoris* can be easily distinguished from the other leuconostocs owing to its poor carbohydrate fermentation capability.

It ferments only glucose, galactose and lactose. Sugars most helpful for the differentiation of *Leuconostoc* species are arabinose, melibiose, trehalose and xylose. For *weissellas*, a battery of sugars has been recommended (Collins et al., 1993) together with some other phenotypic tests. It is essential to use the sugar fermentation tests together with other phenotypic tests.

All *O. oeni* strains ferment lactose, maltose and sucrose (Garvie, 1967a) and this ability can be useful in discrimination from other leuconostocs (Garvie, 1967a; Garvie, 1986). Fermentation profiles of the different *O. oeni* strains vary greatly despite the genetically homogeneous nature of this species.

Cell Wall Composition

Peptidoglycan type analysis has been used as a differentiation criterion, and it is especially useful in recognition of the genus *Weissella*. In this genus, special peptidoglycan types exist.

Electrophoretic Mobility of Enzymes

The number and nature of lactic dehydrogenases (LDHs) and their electrophoretic mobility are species-associated (Gasser, 1970; London, 1976; Garvie, 1980a) and therefore good taxonomic markers. In contrast to the electrophoretic behavior of LDHs from different *Lactobacillus* species, the electrophoretic behavior of NAD-dependent D-LDHs in *Leuconostoc sensu stricto* is quite uniform (Garvie, 1969). The D-LDHs of *L. mesenteroides*, *L. lactis* and *W. paramesenteroides* have identical electrophoretic mobility suggesting similarity of these enzymes. *Oenococcus oeni* D-LDH is, on the other hand, distinct (Garvie, 1969). These D-LDH results correspond with those obtained for dehydrogenases of glucose-6-phospate and 6-phosphogluconate (Garvie, 1975). Their electrophoretic mobility cannot be used for differentiation of the *Leuconostoc sensu stricto* species even though immunology studies have revealed differences among them (Hontebeyrie and Gasser, 1975).

Analysis of Cell Protein and Fatty Acid Patterns

Though routine phenotyping to identify *Leuconostoc* and *Weissella* species is inadequate, numerical analysis of cell protein or fatty acid profiles have proved to be valuable tools in the identification of *Leuconostoc*, *Weissella* and *Oenococcus* species.

TOTAL SOLUBLE CELL PROTEIN PATTERN ANALYSIS BY SDS-PAGE Numerical analysis of total soluble cell protein patterns on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has been used for a large number of different bacterial species (Kersters and De Ley, 1980). Today, it is one of the major tools in polyphasic taxonomy. If this technique is carried out using a set of well-defined reference strains together with a constant approach in the numerical analysis, it enables reliable species identification.

Several reports present protein pattern-based identification data on *Leuconostoc* and *Weissella* species, and on *O. oeni*. Dicks et al. (1990) and (1995) demonstrated that the protein profile groups obtained from different *Leuconostoc* species correspond well with DNA-DNA hybridization data. This technique was used a few years later for differentiating leuconostocs from Argentine raw milk (Fantuzzi et al., 1992) and led to the proposal of a new species *L. argentinum* (Dicks et al., 1993).

Human-originated *Leuconostoc lactis* strains possessing atypical reactions in classical biochemical tests were found to be similar to the *L. lactis* type strain by the means of protein profiles (Barreau and Wagener, 1990). In 1993, Elliott and Facklam (1993) studied the leuconostocs that grew at 37°C and originated from clinical material. From the total of 84 strains, only 9 were not identified to the species level by the protein patterns.

Protein profiles have also been used for the characterization of the LAB from traditional Greek dairy products (Tsakalidou et al., 1994), identification of LAB from Portuguese wines and musts (Patarata et al., 1994), and identification of *O. oeni* from South African fortified wines (Dicks et al., 1995). Villani et al. (1997) used protein profiling for the characterization of *L. mesenteroides* subspecies and reported that the subspecies were not differentiated by this technique. The relatedness of weissellas and leuconostocs has been studied (Dicks, 1995a) as well as that of the atypical leuconostoc-like *Weissella* strains (Tsakalidou et al., 1997).

CELLULAR FATTY ACID ANALYSIS Cellular fatty acid profiles have been used for characterization and identification of *Leuconostoc*, *Weissella* and *O. oeni*.

Eighteen fatty acids were found associated with *O. oeni* (Tracey and Britz, 1987; 1989). The numerical analysis of the fatty acids showed four clusters defined at r = 0.920, with five strains unassigned. On the basis of the amounts of oleic acid and C19-cyclopropane fatty acids, the strains of *O. oeni* could also be distinguished from each other. For the majority of *O. oeni* strains, the result obtained with the cellular fatty acid analysis confirmed the phenotypic relationships.

Shaw and Harding (1989) used cellular fatty acid analysis based clusters together with DNA-DNA reassociation data in the description of *L. carnosum* and *L. gelidum*. The usefulness of this approach for distinguishing different *Weissella* species has also been evaluated (Samelis et al., 1998). The results were overall in good agreement with the previously obtained data. Applying a rapid gas chromatographic method, Samelis et al. could differentiate between *W. viridescens*, *W. paramesenteroides*, *W. hellenica* and some typical arginine-negative *Weissella* isolates from meats on the basis of their cellular fatty acid composition. *Weissella viridescens* synthesized eicosenoic (*n*-C20:1) acid, while the other two species did not. Unlike *W. paramesenteroides*, *W. hellenica* lacked cyclopropane fatty acids with 19 carbon atoms, i.e., dihydrosterculic or lactobacillic acid, whereas *W. viridescens* contained zero to low amounts. Meat isolates identified as *W. viridescens* or *W. hellenica*, shared similar fatty acid profiles with the respective type strains, whereas the "wild" atypical *Weissella* isolates more closely resembled *W. paramesenteroides* and *L. mesenteroides* subsp. *mesenteroides*.

The Use of Ribosomal Genes

IDENTIFICATION APPROACHES BASED ON SPECIFIC PRIMERS AND PROBES Owing to the very high homology of the 16S rRNA genes of leuconostocs (Table 2) and weissellas (Table 4), the percentage values as such cannot be used as criteria for species identification. Therefore, specific primers and probes constructed on the basis of the variable domains of 16S rRNA genes have been designed. Klijn et al. (1991) designed specific DNA probes targeting the variable regions V1 and V3 of 16S RNA genes of LAB including leuconostocs. Also meat-related leuconostocs have been identified using oligonucleotide probes (Nissen et al., 1994). Also, PCR amplification with specific 16S rRNA targeted primers has been used for identification of dairy leuconostocs (Ward et al., 1995). The in situ hybridization method was developed (Sohier and Lonvaud-Funel, 1998) for detection and identification of wine LAB, including *O. oeni*. This approach is based on total genomic DNA probes. For genus *Weissella*, species-specific sequences have been located in the helix 1007/1022 of variable region V6 of 16S rRNA gene (Collins et al., 1993). The spacer sequences of 16S/23S region of some *O. oeni* strains have also been determined (Zavaleta et al., 1997b; Le Jeune and Lonvaud-Funel, 1997).

RIBOTYPING IN SPECIES DETERMINATION Ribotyping (Grimont and Grimont, 1986) is also a good taxonomic tool, and has been mainly used for bacterial typing. Villiani et al. (1997) evaluated ribotyping for the characterization of *L. mesenteroides* but did not find it very useful. On the other hand, Björkroth et al. (1998) found ribotyping excellent for the species identification in a study dealing with a large set of *L. carnosum* strains from ham and the processing environment. This approach was also used in the description of *L. gasicomitatum* (Björkroth et al., 2000) and *W. cibaria* (Björkroth et al., 2002). Ribotyping has also been found to be excellent for *O. oeni* species attribution (Viti et al., 1996). Patterns obtained from closest phylogenetic neighbors were clearly different. Also, Zavaleta et al. (1997a) found very constant ribopatterns associated with *O. oeni* strains.

RFLP OF 16S rRNA GENE *Oenococcus oeni* strains were found to exhibit unique RFLP patterns by *Hae*III digestion of 12 reference strains (Sato et al., 2000). This indicated that the use of 16S rDNA RFLP could be used for the identification of *O. oeni*.

DNA-DNA Hybridization and DNA Base Composition

DNA-DNA hybridization is always the best method for the identification of doubtful strains at the species level. Table 6 shows the levels of DNA-DNA similarity for the species of the genera *Leuconostoc* and *Weissella*, and of *O. oeni*. In some studies, DNA-DNA hybridization has been used to identify spoilage LAB. Cai et al. (1998b) studied *L. gelidum* strains associated with spoilage of ham, and Von Holy et al. (1991) characterized the spoilage flora of vacuum-packaged Vienna sausages. DNA base composition (G+C content) cannot be used for identification purposes owing to the similarity in species' values. G+C-content of DNA from leuconostocs varies from 37 to 45%.

Typing of *Leuconostoc*, *Weissella* and *Oenococcus oeni* by DNA Techniques

PLASMID PROFILING Leuconostocs associated with mesophilic starter cultures have been characterized with plasmid profiling (Johansen and Kibenich, 1992a). Isolates were divided into six groups based on the profiles obtained. All strains contained 2–6 plasmids, and the method was concluded to be suited for *Leuconostoc* typing. Prévost et al. (1995) used plasmid profiling for the characterization of *O. oeni* strains. Six strains were found to contain single cryptic plasmids designated "pUBL01 to pUBL06." The size of these plasmids ranged from 3.9 to 26 kb. The importance of plasmid profiling as a typing

tool has, however, diminished because more stable chromosome-based techniques are now available.

RESTRICTION ENDONUCLEASE ANALYSIS Restriction endonuclease analysis (REA) of the chromosomal DNA together with plasmid profiling were used for strain determination in mixed mesophilic starter cultures (Johansen and Kibenich, 1992a). Results obtained with REA were in harmony with the corresponding plasmid profiles. REA has also been used in a study of genome diversity among strains of *O. oeni* originating from Chianti wines (Viti et al., 1995). In a study dealing with *O. oeni* more generally (Viti et al., 1996), all strains were found to have very similar restriction patterns suggesting that *O. oeni* is genetically a homogeneous species.

RIBOTYPING The discriminatory power of ribotyping is clearly lower than that of the majority of other DNA-based methods. Therefore, it has mainly been found suitable for species identification and not as a strain typing method.

PULSED-FIELD GEL ELECTROPHORESIS Pulsed-field gel electrophoresis (PFGE) typing has been used especially for the characterization of *O. oeni*. PFGE of chromosomal DNA digested with *Not*I or *Sfi*I was used to differentiate individual strains of *O. oeni* (Kelly et al., 1993). In this work, 13 different restriction patterns were obtained from *O. oeni* isolates originating from New Zealand wines undergoing malolactic fermentation. The chromosomes of 41 *O. oeni* strains obtained from different countries have been analyzed using PFGE (Lamoureux et al., 1993). *Not*I, *Sfi*I and *Apa*I and the transverse alternating field electrophoresis (TAFE) PFGE format were used in this study. The use of TAFE with *Apa*I and *Sma*I has also been optimized for *O. oeni* (Daniel et al., 1993). In a third study, 30 *O. oeni* strains were distributed into 20 genomic groups according to PFGE patterns (Tenreiro et al., 1994). *Asc*I, *Not*I, *Sfi*I and *Sma*I were used in this study, and the discriminatory power of these enzymes was considered quite similar. The groups obtained were later found to correspond with RAPD profiles (Zavaleta et al., 1997a). Prévost et al. (1995) determined the genome size of 15 *O. oeni* strains. On the basis of *Eco*RI and *Hin*dIII digestion, the size of the genome varied from 1770 to 1975 kb. Nine- to 23-kb region fragments were found optimal for strain differentiation in this work.

Lactic acid bacteria (LAB) isolated from puto, a fermented rice cake in the Philippines, were characterized using PFGE (Kelly et al., 1995). The microflora was dominated by dextran-producing leuconostocs, and these were differentiated into four groups using PFGE typing together with taxonomic tests. The four groups corresponded to the species *L. mesenteroides* subsp. *mesenteroides*, *L. pseudomesenteroides*, *L. citreum* and *L. fallax*. Using PFGE, Villiani et al. (1997) characterized *L. mesenteroides* strains originating from field grass, natural whey cultures, and water buffalo milk. The discriminatory power of the *Apa*I patterns was concluded to be excellent. PFGE typing was used also for the characterization of *L. carnosum* strains originating from the meat processing industry (Björkroth et al., 1998). *Apa*I and *Sma*I digests divided the strains into 25 different PFGE types, *Apa*I and *Sma*I types being consistent. Similarity analysis revealed a homogeneous cluster that also contained the *L. carnosum* type strain. The *L. carnosum* cluster was clearly distinguished from the clusters of the other meat-associated *Leuconostoc* species. These *L. carnosum* isolates were found to be very homogeneous.

PCR-BASED RANDOM AMPLIFICATION OF POLYMORPHIC DNA Random amplification of polymorphic DNA (RAPD) analysis (synon. amplified ribosomal DNA restriction analysis [ARDRA]-PCR) has been used for the characterization of a diverse strain collection of *O. oeni*. The intraspecific genetic diversity of *O. oeni* was evaluated by RAPD, ribotyping, small-plasmid content, and sequencing of RAPD markers with widespread distribution among the strains (Zavaleta et al., 1997a). The RAPD profiles were strain-specific and discerned two main groups of strains coincident with clusters obtained by macrorestriction typing in previous work (Tenreiro et al., 1994). Ribotyping and the conservation of RAPD markers indicate that *O. oeni* is a relatively homogeneous species. Zapparoli et al. (2000) analyzed the genetic diversity of 60 *O. oeni* strains from different wines by numerical analysis of PFGE patterns with endonuclease *Apa*I, and RAPD-PCR fingerprints with four oligonucleotide primers. Most strains could be identified by distinct RAPD-PCR profiles and associated according to their geographical origin, while only 62% of the strains could be distinguished by the PFGE patterns. Because of its rapidity, RAPD-PCR was considered by Zapparoli et al. (2000) to be a suitable method for typing and monitoring *O. oeni* strains in winemaking.

On the basis of ARDRA-PCR, Villiani et al. (1997) could distinguish two main groups from leuconostocs and weissellas isolated from foliage, milk and whey. The larger group included *L.*

mesenteroides, *L. lactis*, *L. pseudomesenteroides* and some unidentifiable strains; the second one included *L. citreum*, *L. fallax*, *W. paramesenteroides* and some unidentified strains.

Multiplex Approaches

A multiplex polymerase chain reaction (PCR) assay has been developed by Lee et al. (2000) for rapid and reliable identification of *Leuconostoc* species, by using species-specific primers targeted to the genes encoding 16S rRNA. The assay enables the detection and differentiation of *Leuconostoc* species from mixed populations in natural sources and from pure cultures, within 3 h. The assay system consists of a total of 10 primers, two primers from each target species, and comprises two multiplex PCR reactions, one for *L. carnosum*, *L. citreum* and *L. mesenteroides*, and another for *L. gelidum* and *L. lactis*. This PCR assay was used to identify 31 *Leuconostoc* strains isolated from kimchi, and the results showed perfect correlation with the results of a polyphasic method, including 16S rDNA sequencing and DNA-DNA hybridization. The authors concluded that this multiplex PCR is a rapid and reliable method for identification of *Leuconostoc* species in pure cultures or in mixed populations.

Multiplex PCR reactions were used by Yost and Nattress (2001) to characterize populations of LAB associated with meat spoilage. Multiplex PCR was reported to allow the detection of *Carnobacterium* spp., *Lactobacillus curvatus*, *Lactobacillus sakei* and *Leuconostoc* spp. Polymerase chain reaction primers specific for *Carnobacterium* and *Leuconostoc* spp. were created from 16S rRNA oligonucleotide probes and were used in combination with species-specific primers for the 16S/23S rRNA spacer region of *Lactobacillus curvatus* and *Lactobacillus sakei* in the multiplex PCR reactions.

Other Combined Approaches

Molecular diversity of *L. mesenteroides* and *L. citreum*, isolated from traditional French cheeses, was studied by RAPD fingerprinting, 16S rDNA sequencing, and 16S rDNA fragment amplification by Cibik et al. (2000). The combination of RAPD, 16S rDNA sequencing, and 16S rDNA fragment amplification with specific primers allowed the classification of different leuconostocs at the species and strain level. The majority of the 221 strains, which were mainly isolated from traditional French cheeses, were classified as *L. mesenteroides* (83.7%), with *L. citreum* (14%) as the second largest group. According to Cibik et al. (2000), the role of *L.*

citreum in traditional technologies has not been determined before, probably because of the lack of strain identification criteria. Only one strain each of *Leuconostoc lactis* and *L. fallax* were identified, while no *W. paramesenteroides* strains were found. However, the molecular techniques used did not allow the separation of strains of the three *L. mesenteroides* subspecies (*mesenteroides*, *dextranicum* and *cremoris*), and the authors suggested that these subspecies should rather be classified as biovars.

Differentiation of Dextran-producing *Leuconostoc* Strains by a Modified RAPD Protocol

Seven dextran-producing *Leuconostoc* strains were distinguished using a modified RAPD protocol (Holt and Cote, 1998). This protocol incorporates specific primers designed from conserved regions of dextransucrase genes. RAPD profiles showed intraspecies differences among the *L. mesenteroides* strains tested. This modified RAPD protocol aids in the differentiation of polymer-producing leuconostocs. Smith et al. (1998) described a mutant strain (R1510) of *L. mesenteroides* B-1355 which synthesized an insoluble polysaccharide and a slightly soluble polysaccharide when grown in sucrose-containing medium. Glucose or sucrose cultures of this strain produced a single intense band of GTF-1 activity of 240 kDa on SDS gels, and a number of faint, smaller bands. Oligosaccharides synthesized from methyl-α-D-glucoside and sucrose included a trisaccharide with an α(1fwdarw2) glucosidic linkage, not described before. Mutant strains resembling R1510, but producing a single intense band of alternan-sucrase (200 kDa) were also isolated.

Cultivation, Maintenance, and Conservation of Cultures

The general procedures of cultivation, maintenance, and preservation as used for lactobacilli (see The Genera *Lactobacillus* and *Carnobacterium* in this Volume.) are applicable to most leuconostocs and weissellas.

MRS broth or agar is generally used for axenic cultivation, and cultures may be stored as stab cultures (MRS-agar) at 4°C for 1–2 weeks. Vitality may be retained in yeast glucose litmus milk + calcium carbonate for several months (Sharpe, 1981). Information on general cultivation and maintenance of strains has been summarized in Table 8. Juven (1979) recommended the preservation of stock cultures, partly dehydrated, on granular pumice stone for 6–24 months at room

temperature. Lyophilization of *L. mesenteroides* subsp. *mesenteroides* in a solution of native dextran resulted in good long-term survival and retention of the dextran-forming ability (Valakhanovich et al., 1975). *Leuconostoc lactis* and *L. mesenteroides* subsp. *cremoris*, important in starter cultures for their diacetyl-producing ability, were stored at –30°C for 3 months without loss of viability (Oberman et al., 1986). Initial freezing was at –70°C, and sterile milk or cream (18%) gave equally good results as protecting agents. For short-term maintenance of acidophilic leuconostocs, e.g., *O. oeni*, stab cultures in acidic tomato medium at pH 4.8 (Garvie and Mabbitt, 1967c) may be used. Experience over several years has proved the value of cryopreservation at –80°C for practically all LAB. A freshly grown (18–24 h) cell suspension is washed twice in a phosphate buffer (e.g., quarter-strength Ringer's solution), resuspended in the fresh growth medium containing 10–15% sterile glycerol, and distributed in small cryotubes before freezing.

For lyophilization, harvesting of *O. oeni* in the mid-log phase gave maximum viability (Kole et al., 1982). Kole and Altosaar (1984) reported the increase in viability and bile resistance of bile-resistant *O. oeni* strain 44.40 upon lyophilization.

Physiology

Metabolism

As is known for other LAB, growth of leuconostocs and weissellas is dependent on the presence of a fermentable sugar. Glucose is fermented by all species, but fructose is generally preferred. Leuconostocs do not possess a fructose 1,6-diphosphate (FDP)-aldolase. Hexoses are fermented by a combination of the hexose monophosphate system and the phosphoketolase pathway, yielding equimolar amounts of D(–)-lactate, ethanol, and CO_2 (De Moss et al., 1951; Gunsalus and Gibbs, 1952). Being anaerobic by nature, the LAB obtain their metabolic energy by substrate phosphorylation. In addition, three secondary energy transducing processes can contribute to the generation of a proton motive force, i.e., proton/substrate symport as in lactic acid excretion, electrogenic precursor/product exchange as in malolactic and citrolactic fermentation, histidine/histamine exchange, and electrogenic uniport of ions as in malate and citrate uptake in *O. oeni* (Konings et al., 1997). Transport and metabolism of sugars (pentoses, hexoses and disaccharides), organic acids (citrate and malate), and amino acids by *Leuconostoc* spp. have been reviewed by Cogan and Jordan (1994). The effects of internal pH and Mn on growth and metabolism are considerable.

However, in *O. oeni*, this metabolic pathway has not been fully confirmed. Glucose-6-phosphate dehydrogenase and xylulose-5-phosphoketolase are the key enzymes present in all species (Garvie, 1986). In most species, both NAD and NADP may serve as coenzymes of the glucose-6-phosphate dehydrogenase, but in *O. oeni*, only NADP is used (Garvie, 1975). Glucose is phosphorylated and then oxidized to 6-phosphogluconate followed by decarboxylation. The resulting pentose is converted into lactic acid and ethanol. In studying acetaldehyde metabolism by *L. mesenteroides* subsp. *cremoris* under stress conditions, Liu et al. (1997) observed that resting cells converted acetaldehyde to ethanol and acetate. They also showed that low pH, increased levels of salt, and low water activity reduced the rates of acetaldehyde utilization and the formation of ethanol and acetate. Furthermore, almost all leuconostocs tested removed added acetaldehyde in broth cocultures with strains of *Lactococcus lactis* subsp. *cremoris*.

Miranda et al. (1997) proposed a biochemical basis for glucose-induced inhibition of malolactic fermentation in *O. oeni*. They found that 2 mM glucose inhibited malolactic fermentation by 50%, and 5 mM or higher caused a maximum inhibitory effect of ca. 70%. Inhibitory effects similar to that observed with glucose were caused by galactose, trehalose, maltose, whereas ribose and 2-deoxyglucose did not affect the rate of malolactic activity. Addition of fructose or citrate completely reversed the glucose-induced inhibition. High intracellular concentrations of glucose-6-phosphate, 6-phosphogluconate, and glycerol-3-phosphate were detected. The malolactic activity in permeabilized cells or cell extracts was inhibited by glucose-6-phosphate, 6-phosphogluconate, and NAD(P)H, whereas NADP⁺ had no inhibitory effect. The purified malolactic enzyme was strongly inhibited by NADH; by contrast, all previously mentioned metabolites exerted no inhibitory effect, suggesting that NADH was responsible for the inhibition of malolactic activity in vivo (Miranda et al., 1997). Some strains with an oxidative mechanism produce acetate instead of ethanol, and pentose fermentation yields equimolar quantities of D(–)-lactate and acetate. Polysaccharides and most alcohols are not attacked. Malate is converted into L(+)-lactate and CO_2 by strains of *O. oeni* and *L. mesenteroides* subsp. *mesenteroides*. Citrate is metabolized to acetate and lactate. At low pH, however, diacetyl and acetoin may be produced from citrate (Cogan et al., 1981), and all strains examined of *L. mesenteroides* subsp. *cremoris* utilized citrate

(Garvie, 1984). By contrast, acetate and tartrate are not utilized. Increased flavor production occurs at reduced pH, and is especially associated with citrate-lyase-positive strains in the presence of citrate (Speckman and Collins, 1968; Collins and Speckman, 1974; Cogan, 1975), although considerable variations were reported in the amount of diacetyl produced among strains of *L. mesenteroides* subsp. *cremoris* (Walker and Gilliland, 1987). The effect of phenolic acids and anthocyanins on growth, viability and malolactic activity of *O. oeni*, was studied by Vivas et al. (1997), who found that gallic acid and free anthocyanins activated cell growth and also the rate of malic acid degradation. Belguendouz et al. (1997) found differences between *L. mesenteroides* and *Lactococcus lactis* in pH homeostasis and citric acid utilization, and concluded that citric acid allows the maintenance of pH homeostasis in *L. mesenteroides*.

Little is known about the production of biogenic amines by leuconostocs. No tyramine formation was detected in strains of *Leuconostoc* isolated from fresh and vacuum-packaged meat (Edwards et al., 1987) or from *O. oeni* strains isolated from wine (Cilliers and van Wyk, 1985). Choudhury et al. (unpublished results) observed production of tyramine from tyrosine by a strain of *O. oeni* (DSM 20206). Some strains of *L. mesenteroides* subsp. *mesenteroides* and *W. paramesenteroides* were found to produce both tyramine and tryptamine (Bover-Cid et al., 1999).

The ability to degrade phytic acid, an antinutritive factor commonly found in cereals, seems to be rare among LAB such as the leuconostocs. Yet, some *Leuconostoc* strains present in sour dough were reported to have this ability, and *L. mesenteroides* strain 38 was found to improve the Ca and Mg solubility during a 9-h fermentation of phytate-rich whole wheat flour, by degrading phytic acid and producing lactic acid (Lopez et al., 2000).

Nutritional and Environmental Requirements

As most LAB, leuconostocs and weissellas are fastidious and require rich media and complex factors for growth. They prefer facultative anaerobic conditions for growth, and 0.05% cysteine-HCl in the substrate may stimulate growth, while a gas mixture of 19.8% CO_2, 11.4% H_2 and N_2 (ad 100%) will support satisfactory growth on agar media (Dellaglio et al., 1995). Depending on the growth medium, optimum growth for nonacidophilic species ranges between pH 6 and 7, although some strains have been found to grow at pH values >8, in contrast to the lactobacilli (Dellaglio et al., 1995; J. Björkroth, unpublished observations).

Nutritional requirements have been found to be variable among different species and also among different strains of the same species. Like other LAB, leuconostocs and weissellas need a complex medium containing vitamins, nucleotide bases, and amino acids. All species require nicotinic acid, thiamine, biotin and pantothenic acid. *Oenococcus oeni* prefers a gluco-derivative of pantothenic acid (Amachi et al., 1971), which is also known as tomato juice factor (TJF; Garvie and Mabbitt, 1967c), but the degree of dependence varies with different strains and growth conditions. Folic acid is not required for growth of *Leuconostoc* lactis. *Leuconostoc mesenteroides* subsp. *mesenteroides* requires only glutamic acid and valine, whereas the growth of other subspecies and species depends on a variety of amino acids (Garvie, 1967b). *Oenococcus oeni* appears unique in acid and alcohol resistance and in its ability to grow at pH <3.9, and it also differs in some other physiological properties from the leuconostocs. By these features and also by its ability to perform a malolactic fermentation, it is well adapted to the wine environment. Media for the selective enumeration of *O. oeni* often rely on its acidophilic nature. In contrast to other leuconostocs, *L. fallax*, an acid and ethanol tolerant LAB, shows some similarities with *O. oeni*. It is known to be present in sauerkraut, and was reisolated by Middelhoven and Klijn (1997) from exudates of *Gerbera jamesonii*. Strains of *L. fallax* utilized a small number of sugars and showed a remarkable resistance to lactic acid. The final pH in glucose broth was 3.9. *Leuconostoc fallax* was able to grow in the presence of 9.0% (v/v) ethanol and 5.5% salt, but was unable to carry out a malolactic fermentation.

Dextran Production

The leuconostocs were one of the first bacterial groups to be studied (Van Tieghem, 1878), in response to spoilage defects in the sugar industry by slime formation and the resulting commercial losses. This feature appears to be most typical for the subspecies *L. mesenteroides* subsp. *mesenteroides* (previously *L. mesenteroides*), and *L. mesenteroides* subsp. *dextranicus* (formerly *L. dextranicum*). Among some weissellas, and particularly *W. confusa*, dextran production appears to be a common and widespread feature. The effects of particular nutrients on dextransucrase (sucrose: 1,6-α-D-glucan 6-α-D-glucosyltransferase EC 2.4.1.5) production from *L. mesenteroides* NRRL B-512F were studied by Goyal and Kattyar (1997), who observed that an increase in concentration of sucrose to 4%,

resulted in the increase of activity of dextransucrase. Higher enzyme yields were obtained at low yeast extract and high phosphate concentrations. Moreover, the enzyme activity increased by 30% by both peptone and beef extract, while addition of Tween 80 to the medium resulted in enhanced enzyme production and increased activity by 25%. This activity increase was also observed upon addition of sodium fluoride.

Leathers et al. (1997) detected the different polymer-synthesizing *Leuconostoc* sucrases within a polyacrylamide gel using a zymogram. Holt et al. (2001) characterized 12 dextran-producing *Leuconostoc* strains, frequently used in research and industry, according to their antibiotic susceptibilities, carbohydrate fermentation profiles, sucrase activity patterns and plasmid content, and distinguished two groups on the basis of their ability to ferment raffinose and melibiose.

Kitaoka and Robyt (1999) studied the mechanism of action of *L. mesenteroides* B-512FMC dextransucrase, and showed this enzyme to catalyze the synthesis of dextran from sucrose with a catalytic rate constant (k[cat]) of 641 s^{-1}, and the transfer of D-glucose from sucrose to maltose with a k(cat) of 1070 s^{-1}. This enzyme was also found to catalyze two new reactions in the absence of sucrose, using dextran as the substrate. These reactions are based on consistent "ping-pong/bi-bi" kinetics. Arguello Morales et al. (2001) investigated cellobiose as acceptor, and compared the oligosaccharides synthesized by alternansucrase (EC 2.4.1.140) from *Leuconostoc mesenteroides* NRRL B-23192 to those obtained with dextransucrase from *L. mesenteroides* NRRL B-512F. Overall, oligosaccharide synthesis yield reached 30 and 14% with alternansucrase and dextransucrase, respectively, indicating that alternansucrase is more efficient than dextransucrase for cellobiose glucosylation.

Biogenic Amines

A special screening medium, developed for assaying LAB for their ability to form biogenic amines (BA), indicated particular ability of strains of *L. mesenteroides* subsp. *mesenteroides* to decarboxylate tyrosine, while one strain of each of the subspecies *L. mesenteroides* subsp. *cermoris* and *L. mesenteroides* subsp. *dextranicus*, and also of *O. oeni*, *W. confusa* and *W. viridescens*, were found negative (Bover-Cid and Holzapfel, 2000). This feature seems to vary among strains of the bacterial groups under discussion, and some positive strains of *L. mesenteroides* subsp. *cremoris* and *W. paramesenteroides* have been reported earlier to produce tyramine and also tryptamine (Bover-Cid and Holzapfel, 2000). A direct PCR detection test, allowing an early detection of histamine-producing bacteria, indeed showed that the presence of histidine decarboxylating (HDC+) bacteria was not rare. Almost half of 118 tested wines were found to contain bacteria carrying histidine decarboxylase, and all the HDC+ strains were shown to belong to *O. oeni* (Coton et al., 1998b).

Genetics

Genomic Organization of the *Leuconostoc* Group of Organisms

The genome analysis of leuconostocs and related organisms is in an early stage compared to some other lactic acid bacterium genera, such as the genus *Lactococcus*. In respect to genome organization, a physical map of the genome of *O. oeni* PSU-1 containing the location of some genetic markers has been established (Zé-Zé et al., 1998) and the genome sizes of some *Leuconostoc* species have been determined. The genomes of three *L. mesenteroides* strains were reported to have sizes between 1.76 and 2.03 Mbp (Tenreiro et al., 1994). *Leuconostoc citreum* (two strains) yielded 1.75–1.84 Mbp, *L. gelidum* strain L4026 2.17 Mbp and *L. pseudomesenteroides* strain L4027 2.05 Mbp (Tenreiro et al., 1994).

The sizes of the genome of 42 *O. oenis* strains have been determined, the values obtained ranged from 1.78 to 2.1 Mbp (Kelly et al., 1993; Lamoureux et al., 1993; Tenreiro et al., 1994; Prévost et al, 1995; Zé-Zé et al., 1998). Both the mapping of the *O. oeni* genome and all size evaluations were performed using pulsed-field gel electrophoresis technique employing macrorestriction analysis of genomic DNA.

Oenococcus oeni PSU-1 Map

A physical map of *O. oeni* PSU-1 has been constructed (Zé-Zé et al., 1998). This work was based on the use of *Asc*I, *Fse*I, *Not*I and *Sfi*I mapping of the chromosome. The size of the chromosome was estimated to be 1857 kb and it was found to contain two *rrn* operons showing the typical 5′-*rrs-rrl-rrf*-3′ organization found in the majority of bacteria. Oenococcal genes *alsS* and *alsD* (α-acetolactate synthase and decarboxylase) (Garmyn et al., 1996), *mleA* (malolactic enzyme; Labarre et al., 1996a), and *mir* (mitomycin resistance) were mapped in the chromosome. Two phage attachment sites and sequences of IS*1165*-like elements were also located on the physical map.

The physical and genetic maps of the *O. oeni* strains GM and PSU-1, which represent two genomic divergent groups on the basis of mac-

rorestriction and ribotyping analysis have also been compared (Zé-Zé et al., 2000). All the recognition sites of the restriction enzymes *Asc*I, *Ceu*I, *Fse*I, *Not*I and *Sfi*I were found to be located in both chromosomes, and the position of 26 genetic markers, including two *rm* operons and 14 new putative oenococcal genes, were allocated to the restriction fragments generated by the five enzymes. The comparative analysis of *O. oeni* GM and PSU-1 genomes revealed extensive conservation of loci order. No evidence for major genomic rearrangements was found. The genomic conservation between the two strains was in agreement and suggested homogeneity within the species (Zavaleta et al., 1997a and 1997b).

Genetic Tools for Leuconostocs and Related Organisms

Few methods designed specially for *Leuconostoc-Weissella* have been designed. A transformation method for *W. paramesenteroides* (David et al., 1989) has been published. For a controlled gene expression system suitable also for *Leuconostoc* species, a transferable nisin-inducible expression cassette has been developed (Kleerebezem et al., 1997). It is a transferable dual plasmid system based on the nisin autoregulatory process. Introduction of these plasmids allows nisin-inducible gene expression in *Leuconostoc* spp.

Related to the genetic tools and *O. oeni*, very little has been published, the only work being an electroporation-based transformation system (Dicks, 1994). From the three plasmid vectors tested in this work, only pGK13 was expressed and the electroporation conditions of 2000 V/cm, 25 µF and 200 ohm yielded transformation efficiencies of 1×10^3.

Plasmids in *Leuconostoc*, *Weissella* and *Oenococcus oeni*

The first genetic studies were dealing mainly with the existence of plasmid DNA in leuconostocs and weissellas (O'Sullivan and Daly, 1982; Orberg and Sandine, 1984). Later, some of the plasmids have been studied in more detail, resulting in the association of them with certain properties, such as citrate and carbohydrate metabolism or bacteriocins. Nevertheless, the majority of the plasmids of *leuconostocs* and related organisms have no known function. The cryptic plasmid pCI411 from *L. lactis* 533 has been cloned and sequenced (Coffey et al., 1994). Its genetic organization was deduced and the results suggested a rolling circle replication mechanism.

The occurrence of plasmids in a variety of *O. oeni* strains (Orberg and Sandine, 1984; Janse et al., 1987) has been demonstrated. Only few *O.*

oeni plasmids have been characterized to the sequence level. Analysis of the nucleotide sequence of the pLo13 plasmid (Fremaux et al., 1993) showed that it probably replicates through a rolling circle mechanism and belongs to the pC104 family (Novick, 1989), despite its low similarity to the other members of this family. Zuniga et al. (1996) determined the complete sequence of a cryptic plasmid p4082. The analysis revealed five open reading frames (ORF) all located on the same strand. No similarity was found between the sequence and the European Molecular Biology Laboratory (EMBL) GenBank database-deposited sequences. However, a putative ATP-binding motif was found in ORF2, and a more detailed analysis of it suggested the possible encoding of a DNA-dependent ATPase. Brito and Paveia (1999) applied a large-scale isolation technique to screen 30 *O. oeni* strains for extrachromosomal DNA. Most strains were found to contain large plasmids (ca. 40 kb) with small plasmids (2.5–4.5 kb) only in 6 of these strains. The circular nature of the large plasmids was assessed by electrophoresis in ethidium bromide continuous gradient gels, and their different conformations could be distinguished by three run types of PFGE. Southern hybridization suggested low extraction yields of possibly low-copy-number plasmids to be related to the generation of the open circular (OC) conformation as a result of nicking during cell lysis. Large plasmids seem to occur more frequently in *O. oeni* than expected, while spontaneous curing of these elements also appears to occur.

16S-23S and 23S-5S rRNA Intergenic Spacer Regions in *Leuconostoc* and *Oenococcus oeni*

The 16S-23S (spacer-1) and 23S-5S (spacer-2) rRNA intergenic spacer regions of *L. lactis*, *L. mesenteroides*, *L. mesenteroides* subsp. *dextranicum* and *L. mesenteroides* subsp. *cremoris* have been sequenced together with the 23S rRNA genes of *Leuconostoc lactis*, *L. mesenteroides*, and *L. mesenteroides* subsp. *dextranicum* (Nour, 1998). The RNase III-like and RNase E processing sites, as well as putative antitermination signals, were identified within the spacer regions. A single *tRNA-Ala* gene without the 3′-terminal CCA sequence was found in spacer-1 regions. Secondary structure models showed interactions between the two spacer-regions of leuconostocs. For all strains studied, spacer-1 and spacer-2 were highly conserved and therefore could not be directly used for strain typing. Sequence information on 23S rRNA genes from *Leuconostoc* species allowed the determination of regions that can be used as targets for diagnostic probes and amplification primers. Comparative analysis

of the secondary structures of variable helical elements revealed that restriction analysis of 23S rRNA variable regions appeared to be sufficient for the search for species-specific signatures. Only one form of the rRNA operons was found present in leuconostocs and the direct linkage between the three species of rRNA genes was demonstrated. The genes were organized as follows: 5′-16S rRNA – spacer-1 – tRNA-Ala-23S rRNA – spacer-2 – 5S rRNA-3′.

The intergenic regions of three *O. oeni* strains have been studied (Le Jeune and Lonvaud-Funel, 1997). The 16S/23S rRNA intergenic spacers were amplified, sequenced and the sequences compared. The spacer sequence was highly conserved. Inside this spacer, a *tRNA-Ala* gene was discovered. It contained an 18-bp sequence stretch that is conserved in all tRNA genes.

Insertion Sequences in the Genus *Leuconostoc*

Three different insertion sequence (IS) elements, IS*1165*, IS*1070* and IS*1297*, have been associated with leuconostocs. IS*1165* was isolated and characterized from *L. mesenteroides* subsp. *cremoris* DB1165 strain (Johansen and Kibenich, 1992a). The size of this element is 1553 bp, and it has imperfect inverted repeat ends and contains an open reading frame (ORF) of 1236 bp. It was not associated with any previously described IS sequences. The copy number in *L. mesenteroides* subsp. *cremoris* varied from 4 to 13. Also, IS*1165* or closely related elements were found in *O. oeni*, *L. lactis*, *pediococci*, *Lactobacillus helveticus*, but not in *Lactococcus*.

IS*1070* was identified and characterized from the lactose plasmid of *L. lactis* NZ6009 (Vaughan and de Vos, 1995a). The 1027-bp sequence of it contains partially matched inverted repeats and one ORF. Protein homology of the deduced 305-amino-acid sequence demonstrated homology to other bacterial IS-transposases. Fifteen IS*1070*-like sequences were detected in the genome of the parent strain and five of these sequences were located in plasmids.

The third IS element, IS*1297*, is an ISS*1*-like insertion sequence (Ward et al., 1996). No direct repeats were found immediately flanking IS*1297*; however, direct repeats were present approximately 60 bp on either side of the insertion site. IS*1297* contained a major ORF of 681 bp, encoding a putative 226-amino-acid protein with 96.5% homology to the presumed transposase of ISS*1*. An overlapping ORF of 174 bp in the same orientation was also present. A putative ORF in the opposite orientation to the transposase ORF, which has been shown in some iso-ISS*1* elements, was not present in IS*1297*. Also, IS*1297*

was shown to hybridize with other dairy *Leuconostoc* strains.

Genetics of Citrate and Lactate Metabolism in Leuconostocs

The citrate permease determinant (*citP*) has been demonstrated to be plasmid encoded in several *Leuconostoc* strains (Tsai and Sandine, 1987a; Vaughan et al., 1995b). Cloning and nucleotide sequence analysis of *L. lactis* NZ6070 *citP* revealed almost complete identity to lactococcal *citP*. Bekal-Sadja et al. (1998) purified a citrate lyase from *L. mesenteroides* and characterized the gene cluster involved. The 2.7-kb gene cluster encoding citrate lyase of *L. mesenteroides* is organized in three ORFs, *citD*, *citE* and *citF*, encoding, respectively, the three citrate lyase subunits γ (acyl carrier protein [ACP]), β (citryl-S-ACP lyase), and α (citrate-acetyl-ACP transferase). The gene *citC* encoding the citrate lyase ligase (EC 6.2.1.22) was localized in the region upstream of *citD*. Protein comparisons showed similarities with the citrate lyase ligase and citrate lyase of *Klebsiella pneumoniae* and *Haemophilus influenzae*.

The instability of Lac+- and Cit+-phenotypes has been investigated in *L. mesenteroides* subsp. *cremoris* ATCC 19245 and in 4 strains of *L. mesenteroides* subsp. *dextranicum* (Fantuzzi et al., 1991. The 2 phenotypes were linked, respectively, to a 14-MDa and a 34-MDa plasmid in *L. mesenteroides* subsp. *cremoris* ATCC 19245. In *L. mesenteroides* subsp. *dextranicum* the character Lac+- was linked to a 28-MDa plasmid, while the Cit+-phenotype was stable.

A 16-kb *Bam*HI fragment of the lactose plasmid pNZ63 from *L. lactis* NZ6009 has been cloned in *Escherichia coli* MC1061 resulting in the expression of a functional β-galactosidase (David et al., 1992). Deletion and complementation analysis showed that the coding region for β-galactosidase was located on a 5.8-kb *Sal*I-*Bam*HI fragment. Nucleotide sequence analysis determined that this fragment contained two partially overlapping genes, *lacL* (1,878 bp) and *lacM* (963 bp) encoding proteins with calculated sizes of 72, 113 and 35,389 Da, respectively. The *L. lactis* β-galatosidase was overproduced in *E. coli*. Two new proteins with M-rs of 75,000 and 36,000 appeared and the N-terminal sequences of these proteins corresponded to those deduced from the *lacL* and *lacM* gene sequences. Mutation and deletion analysis showed that *lacL* expression is essential for *lacM* production and that both the *lacL* and *lacM* genes are required for the production of a functional β-galactosidase in *E. coli*. The deduced amino acid sequences of the LacL and LacM proteins

showed considerable identity with the sequences of the N- and C-terminal parts, respectively, of β-galactosidases from other LAB or *E. coli*. DNA sequence and protein sequence alignments suggested that the *L. lactis lacL* and *lacM* genes have been generated by an internal deletion in an ancestral β-galactosidase gene.

The gene encoding the lactose transport protein (LacS) of *L. lactis* NZ6009 has been cloned from its native lactose plasmid, pNZ63 (Vaughan et al., 1996). Nucleotide sequence analysis revealed an ORF with the capacity to encode a protein of 639 amino acids, the sequence of which had limited but significant identity to the lactose transport carriers (*lacS*) of *Streptococcus thermophilus* (34.5%) and *Lactobacillus bulgaricus* (35.6%). This similarity was present both in the amino-terminal hydrophobic carrier domain and in the carboxy-terminal enzyme IIA-like regulatory domain. Preceding the *lacS* gene was a small ORF in the same orientation encoding a deduced 95-amino-acid protein with a sequence similar to the amino-terminal portion of β-galactosidase I from *Bacillus stearothermophilus*. The *lacS* gene was separated from the downstream β-galactosidase genes (*lacL* and *lacM*) by 2 kb of DNA containing an IS*3*-like insertion sequence, which was novel for *lac* genes in comparison with that in other LAB. The *lacS* gene was expressed both in a *lacS* deletion derivative of *S. thermophilus* and in a pNZ63-cured strain, *Leuconostoc lactis* NZ6091. Substantial uptake of radiolabeled lactose or galactose was observed with *Leuconostoc lactis* or *S. thermophilus* plasmids harboring an intact *lacS* gene. Galactose uptake observed in NZ6091 suggests the presence of at least one more transport system for galactose in *Leuconostoc lactis*.

Genetics of Dextransucrases of Leuconostocs

The coding region for a *L. mesenteroides* NRRL B-1299 dextransucrase gene (*dsrA*) has been isolated and sequenced (Monchois et al., 1996). The sequence of the *dsrA* was found to consist of an ORF of 4870 bp coding for a 1290-amino-acid protein. The amino acid sequence exhibited a high similarity with streptococcal glucosyl transferases (GTF). The two domains previously described in GTFs are conserved in this dextransucrase (DSRA): an N-terminal conserved domain and a C-terminal domain composed of a series of repeats. The dextran produced appeared to be composed of 85% α(1-6) and 15% α(1-3)-linkages, and the oligosaccharides synthesized in the presence of maltose were mainly composed of α(1-6)-linkages. The coding region for another *L. mesenteroides* NRRL B-

1299 dextransucrase gene (*dsrB*) has also been isolated and sequenced (Monchois et al., 1998). The nucleotide sequence of the *dsrB* gene was determined and found to consist of an ORF of 4521 bp coding for a 1507-amino-acid protein. The amino acid sequence is very close to that of DSRA. Like the product of DSRA catalysis, the dextran produced appeared to be composed of only α(1-6)-glucosidic bonds, and the oligosaccharides synthesized in the presence of acceptor maltose were also composed of α(1-6)-linked glucosyl residues in addition to the maltosyl residue. Monchois et al. (1997) have also cloned the genes (*dsrS*) encoding dextransucrase DSRA.

A sucrase gene from *L. mesenteroides* has been cloned and expressed in *E. coli* (Holt and Cote, 1997). The cloned enzyme did not show dextransucrase or sucrose phosphorylase activity. High-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) analyses of the sucrase products indicated the presence of fructose and glucose in equimolar amounts. Isopropyl β-D-thiogalactopyranoside (IPTG) induction did not increase sucrase activity in *E. coli*, indicating that the cloned gene may be transcribed from its own promoter. This is apparently the first sucrase cloned from *L. mesenteroides* that has invertase activity.

DNA (RAPD) protocol that incorporates specific primers designed from conserved regions of dextransucrase genes has been developed for the characterization of the dextran-producing *L. mesenteroides* strains (Holt and Cote, 1998). RAPD profiles showed intraspecies differences among the *L. mesenteroides* strains tested. This modified RAPD protocol was developed for differentiation of polymer-producing leuconostocs, which have previously been distinguished by time-consuming analyses of the synthesized dextrans.

Bacteriocin Genetics in the Genus *Leuconostoc*

A majority of the studies on bacteriocin production has focused on the search of bacteriocin-producing strains, characterization of the inhibitory spectrum, and purification of the bacteriocin. The early genetic studies were mainly associated with plasmids, but more detailed studies have been done regarding leucocin A (Hastings et al., 1991; Van Belkum and Stiles, 1995), leucocin B (Felix et al., 1994), leucocin J (Choi et al., 1999), and mesentericin Y 105 (Fremaux et al., 1995; Biet et al., 1998). Also the conjugal transfer of several plasmids (Tsai and Sandine, 1987b; Tsai and Sandine, 1987a) has been demonstrated.

LEUCOCIN A Leucocin A-UAL 187 is a plasmid-mediated bacteriocin produced by *L. gelidum* UAL 187 (Hastings et al., 1991). The operon consists of a putative upstream promoter, a downstream terminator, and two open reading frames (ORF) flanked by a putative upstream promoter and a downstream terminator. The first ORF downstream of the promoter contains 61 amino acids and is the leucocin structural gene, consisting of a 37-amino-acid bacteriocin and a 24-residue N-terminal extension. No phenotypic expression of the bacteriocin was evident in several LAB that were electrotransformed with pNZ19 containing the 2.9-kb cloned fragment of the leucocin A plasmid. When a 1-kb *Dra*I-*Hpa*I fragment containing this operon was introduced into a bacteriocin-negative variant (UAL187-13), immunity but no leucocin production was detected (Van Belkum and Stiles, 1995). Leucocin production was observed when an 8-kb *Sac*I-*Hind*III fragment of the leucocin plasmid was introduced into *L. gelidum* UAL187-13 and *Lactococcus lactis* IL1403. Nucleotide sequence analysis of this 8-kb fragment revealed the presence of three ORFs in an operon upstream of and on the strand opposite from the leucocin structural gene. The first ORF (*lcnE*) encodes a putative protein of 149 amino acids with no apparent function in leucocin A production. The second ORF (*lcnC*) contains 717 codons that encode a protein homologous to members of the HlyB family of ATP-binding cassette transporters. The third ORF (*lcnD*) contains 457 codons that encode a protein with marked similarity to LcnD, a protein essential for the expression of the lactococcal bacteriocin lactococcin A. Deletion mutations in *lcnC* and *lcnD* resulted in loss of leucocin production, indicating that LcaC and LcaD are involved in production and translocation of leucocin A. The secretion apparatus for lactococcin A did not complement mutations in the *lcnCD* genes to express leucocin A in *Lactococcus lactis*. However, lactococcin A production was observed when the structural and immunity genes for this bacteriocin were introduced into a leucocin producer of *L. gelidum* UAL187, indicating that lactococcin A could be exported by the leucocin A secretion machinery.

LEUCOCIN B *Leuconostoc carnosum* Ta11a produces a bacteriocin designated "leucocin B-Ta11a" (Felix et al., 1994). An 8.9-MDa plasmid in *L. carnosum* Ta11a was hybridized to a 36-mer-oligonucleotide-probe (JF-1) that was homologous to leucocin A-UAL187. The presence of two ORFs was revealed. The first, ORF1, codes for a protein of 61 amino acids comprising a 37-amino-acid bacteriocin, and was determined to be the leucocin B-Ta11a structural gene by

virtue of its homology to leucocin A-UAL 187 (Hastings et al., 1991). The 24-amino-acid N-terminal extension, however, differed from that of leucocin A-UAL187 by seven residues. The predicted protein of the ORF2 has 113 amino acids and is identical with the amino acid sequence of the cognate ORF of the leucocin A-UAL 187 operon.

Leuconostoc sp. LAB145-3A has been reported to produce leucocin B (Ahn and Choi, 1997). The strain has two residential plasmids of sizes 23 and 48 kb. A comparison of plasmid profiles revealed that the 23-kb plasmid, pCA23, was responsible for bacteriocin production and immunity.

LEUCOCIN J *Leuconostoc* sp. J2, isolated from naturally fermented kimchi, produces leucococin J (Choi et al., 1999). This bacteriocin exhibited an inhibitory activity against several lactic acid bacteria (LAB) and some food borne pathogens. The antimicrobial substance was secreted into the medium during the late log phase. It appears to be proteinaceous since its activity was completely inactivated by a range of proteolytic enzymes, and it was also relatively heat stable. The apparent molecular mass of partially purified bacteriocin, as indicated by activity detection after Tricine-SDS-PAGE, was 2.5–3.5 kDa. Phenotypic expression of the bacteriocin production was detected in transformants harboring pULBJ5.5.

MESENTERICIN Y10^5 Mesentericin Y10^5 is a small non-lantibiotic bacteriocin (class II) encoded within a 35-kb plasmid from *L. mesenteroides* Y10^5 (Fremaux et al., 1995). The mesentericin Y10^5 structural gene, *mesY*, encodes a precursor of the bacteriocin with a 24-amino-acid N-terminal extension ending with a Gly-Gly motif upstream of the cleavage site. Four other putative genes are associated with *mesY* within two divergent putative operons. In addition to *mesY*, the first putative operon was predicted to encode a protein similar to that encoded by ORF 2 in the leucocin A operon, whose function remains to be elucidated. The second putative operon contains three ORFs, two of which, *mesY* and *mesY* encode proteins that resemble ATP-dependent transporters and accessory factors, respectively. For three other class II bacteriocin systems (lactococcin A, pediocin PA-1, and colicin V), these proteins have been shown to be involved in bacteriocin secretion independently of the general sec-dependent secretion pathway. The last putative gene (*mesC*) did not resemble any previously characterized gene. The maturation and secretion functions dedicated to lactacin F (another class II bacteri-

ocin) seem to be efficient for mesentericin Y105 as well.

To investigate heterologous expression systems capable of producing mesentericin Y10S in various hosts, two different secretion vectors were constructed (Biet et al., 1998). One of them, containing the mesentericin Y105 structural gene fused to the segment encoding the divergicin A signal peptide, was introduced into *E. coli*, *Leuconostoc* spp. and *Lactococcuus* spp. In *E. coli*, mesentericin Y105 production was linked to a putative periplasmic toxicity. The mesentericin Y105 precursor was also produced in *E. coli*. It was demonstrated that this pre-bacteriocin exhibited some antagonistic activity. In *Leuconostoc* spp., the production of mesentericin Y105 was enhanced via the dedicated transport system (DTS) compared to the general secretion pathway.

Genetics of Oenological Properties of *Oenococcus oeni*

BACTERIOPHAGES OF *OENOCOCCUS OENI* AND THE MALOLACTIC FERMENTATION   The presence of bacteriophages of *O. oeni* was first demonstrated by Sozzi et al. (1976) in Swiss wines. This was followed by similar observations in Australian (Davis et al., 1985a) and South African wines (Nel et al., 1987). The association of phages with some abnormalities in malolactic fermentation has also been described (Sozzi et al., 1982; Davis et al., 1985a; Henick-Kling et al., 1986b), and the importance of lysogenic *O. oeni* strains as a source of phages has been demonstrated (Arendt et al., 1991).

To detect phages, Tenreiro et al. (1993) used mitomycin C treatment to induce 29 strains originating mainly from Portugal. In a total of 19 strains, phages were present and the analysis of their host-range suggested that phage typing would be of use in *O. oeni*. The modern DNA techniques have, however, replaced the need for phage typing as a strain characterization tool. Lysogeny of *O. oeni* was studied also by Poblet-Icart et al. (1998). Mitomycin C induced lysogeny in 45% of the total 167 strains tested. The phages were all found to have classical morphology, an isometric head and a long striated tail. The sensitivity of bacteria to phages was strain-dependent and all lysogenic strains were resistant to infection by the temperate phage they released. The release of phages did not affect the growth of *O. oeni* strains. In general, the strains that were very sensitive to infection were suspected to be prophage-free.

Wine composition may affect the infective capacity of phages. It has been demonstrated that phages are inactivated by some factors of wine, such as low pH and sulfur dioxide (Davis et al., 1985a; Henick-Kling et al., 1986a). This suggests that phage attack may not be the most important reason for problems in malolactic fermentation. The results of Poblet-Icart et al. (1998) showed also that lysogenic malolactic starter cultures can be used, although they advised taking phage problems into account. The use of mixed cultures with different phage sensitivities or the use of lysogenic strains was recommended (Poblet-Icart et al., 1998).

A 32-kb *Eco*RI-*Hin*dIII DNA fragment of *O. oeni* bacteriophage L10 has been cloned and sequenced (Sutherland et al., 1994). Eleven possible ORFs located on the same strand were detected by a computer-assisted analysis. In vitro transcription/translation analysis yielded five prominent proteins that were correlated with ORFs by their sizes and expression from deleted clones. Significant homologies with other known sequences were not found.

Santos et al. (1996) characterized the temperate bacteriophages of *O. oeni* and found evidence for two prophage attachment sites in the genome of PSU-1 starter strain. This result was confirmed by the physical map generated of PSU-1 (Zé-Zé et al., 1998). The 17 phages studied all had cohesive end termini and sizes ranging from 36.4 to 40.9 kb. Restriction endonuclease analysis grouped them into 6 groups. Lysogenization of a spontaneous phage-cured PSU-1 was obtained with 16 phages, and the analysis of the lysogeny showed that interaction with the host DNA occurred in one or 2 sites in the chromosome. Another integration system, the system of phage 10MC in the LOF 111 *O. oeni*, has also been studied (Gindreau et al., 1997). A 1456-bp fragment of phage DNA was cloned and sequenced and an ORF showing sequence homology with several temperate phage integrases was located upstream of the phage attachment site. The same bacterial attachment site located in the *tRNAleu* gene was found in all 15 *O. oeni* strains, and it was considered to be involved in the π10MC chromosomal integration.

THE *MLE* LOCUS AND *ALSD* GENE ASSOCIATED WITH OENOLOGICAL PROPERTIES   The most important technological characteristics of *O. oeni* are related to its ability to perform malolactic fermentation in wines. This fermentation is secondary to alcohol fermentation and it decreases acidity, enhances organoleptic properties, and prevents wine spoilage by the conversion of L-malate into L(+)-lactate and CO_2. A 3.4-kb fragment carrying two overlapping ORFs (*mleA* and *mleP*) encoding the malolactic enzyme and malate permease has been sequenced (Labarre et al., 1996b). Existence of a dicistronic transcriptional unit encompassing the *mleA* and *mleP* genes has been demonstrated, and the transcrip-

tion start site of the operon has been determined (Labarre et al., 1996a). A third ORF encoding a protein belonging to the LysR-type regulatory protein family was found upstream of the *mleA* gene. It was similar to the activator protein MleR of *L. lactis* (Renault et al., 1989). No regulation, however, of the malolactic enzyme by L-malate was effective under the experimental conditions. Primers based on the *mleA* gene have been designed enabling the detection of *O. oeni* in must and wine (Zapparoli et al., 1998).

The *alsD* gene encoding α-acetolactate decarboxylase has been characterized (Garmyn et al., 1996). The nucleotide sequence of *alsD* gene encodes a putative protein of 239 amino acids showing significant homology with other bacterial α-acetolactate decarboxylases. Upstream from *alsD* gene lies an ORF (*alsS*), which is highly similar to bacterial genes encoding catabolic α-acetolactate synthases. Northern analyses indicated a presence of a 2.4-kb dicistronic transcript of *alsS* and *alsD* suggesting that they were organized in a single operon.

Histidine Decarboxylase of *Oenococcus oeni* 9204

The *hdc* gene encoding histidine decarboxylase has been cloned and sequenced (Coton et al., 1998a). It encodes a single polypeptide of 315 amino acids. This enzyme is responsible for production of histamine, a biogenic amine, which may occur in wines. On the basis of the *hdc* gene sequence, Le Jeune et al. (1995) designed a primer set and probes for detection of histidine decarboxylating LAB, including *O. oeni*.

Stress and Heat Shock Response in *Oenococcus oeni*

Multiple stress factors, such as heat, ethanol and acidity induce the formation of an 18-kDa polypeptide named "Lo 18" (Guzzo et al., 1997). It has been purified and its N-terminal sequence has been determined (Guzzo et al., 1997). The gene encoding Lo 18 *hsp18* encodes a polypeptide of 148 amino acids (Jobin et al., 1997). It is significantly identical with small heat shock proteins of the α-crystallin family. The promoter region of *hsp18* was also identified, and it exhibited high similarity to the consensus promoters of Gram-positive bacteria as well as *E. coli*. *hsp18*, which consists of a unique transcription unit of 0.6 kb. Its expression was considered to be controlled at the transcriptional level, and it was found to associate peripherally with the bacterial membrane. Derré et al. (1999) characterized a novel protein regulator, CtsR, which regulates stress and heat shock response by controlling *clp* and molecular chaperone gene

expression in Gram-positive bacteria. The target sequence of CtsR was also found upstream from *hsp18* of *O. oeni*.

Ecology

By their common physiological characteristics, most strains of *Leuconostoc* and *Weissella* are well adapted to food substrates, both of plant and animal origin (Holzapfel and Schillinger, 1992). They share these habitats with other LAB, and particularly with lactobacilli and pediococci, generally showing complex nutritional requirements, oxygen tolerance, and preference of microaerophilic conditions. They are typically associated with food fermentations, and are frequently predominant in the early stages of fermentation, where particularly *L. mesenteroides* subsp. *mesenteroides* and *W. paramesenteroides* play an important role in initiating the desired fermentation. The phyloplane of many plants appears to be a typical "natural" habitat. "Spontaneous" plant materials (i.e., present in plant materials without deliberate inoculation with a starter culture) are exemplified by sauerkraut, kimchi, cucumbers, carrots and also silage (Dellaglio and Torriani, 1986; Daeschel et al., 1987; Buckenhueskes). Their "natural" association with plant food products has in fact been recognized long ago (Mundt et al., 1967), while their causative role in expolysaccharide production at sugar mills, has been studied together with the first scientific description of this genus >125 years ago (Van Tieghem, 1878). *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Leuconostoc lactis* are also associated with milk and milk fermentations, and more recently, *L. argentinum* was described to be associated with Argentinian raw milk (Dicks et al., 1993). Leuconostocs are commonly found in meat and meat products, although mainly the psychrotolerant species *L. carnosum* and *L. gelidum* tend to dominate these associations (Holzapfel, 1998). *Leuconostoc gasicomitatum* is also a psychrotolerant species, and it is located in the same phylogenetic branch with *L. carnosum* and *L. gelidum* on the basis of 16S rDNA analysis (Björkroth et al., 2000).

Antibiotic Resistance and Clinical Isolates

Several workers have reported the isolation of vancomycin-resistant leuconostocs from clinical sources (Coovadia et al., 1987; Coovadia et al., 1988; Horowitz et al., 1987; Hardy et al., 1988; Isenberg et al., 1988; Luetticken and Kunstmann, 1988; Rubin et al., 1988; Ruoff et al.,

1988; Wenocur et al., 1988). The trait of vancomycin resistance alarmed physicians towards the end of the 1980s (Horowitz et al., 1987; Horowitz et al., 1989) and leuconostocs were even considered to be emerging pathogens. However, the genus *Leuconostoc* was shown to be intrinsically resistant to vancomycin. The mechanism of their resistance is completely different from the transferable vancomycin resistance of enterococci. Leuconostocs associated with human sources have been characterized by Barreau and Wagener (1990). Leuconostocs from clinical sources were associated with infections such as purulent meningitis (Coovadia et al., 1987; Coovadia et al., 1988), odontogenic infection (Wenocur et al., 1988), catheter-associated infection (Hardy et al., 1988), and bacteremia or septicemia (Bernaldo de Quiros et al., 1991; Ling, 1992; Petters et al., 1992; Elliott and Facklam, 1993). Other examples of initial clinical diagnosis were less frequent, and include subacute bacterial endocarditis, pneumonia, AIDS, liver abscess, urinary tract infection, metabolic acidosis, multiple myeloma, prostate cancer, surgical wound infection, and peritonitis (Facklam and Elliott, 1995). A rare case of pleural empyema caused by *Leuconostoc* spp. was reported by Borer et al. (1997) for a patient with characteristic predisposing factors, such as a serious underlying disease, previous vancomycin therapy, and thoracic access device.

Classification of the vancomycin-resistant, Gram-positive cocci with leuconostocs has been supported by comparative taxonomic studies involving DNA hybridization studies (Farrow et al., 1989). One reason (amongst others) clinical *Leuconostoc* strains were previously misclassified as unidentified *Enterococcus* spp. was because up to 31% of these strains reacted with the group D antiserum (Facklam and Elliott, 1995). Recognition of the fact that some leuconostocs may indeed act as opportunistic pathogens resulted in the classification of some of these clinical isolates into the two species, *L. citreum* and *L. pseudomesenteroides* (Farrow et al., 1989). Out of 101 clinical isolates received by the United States Centers for Disease Control and Prevention (CDC), the largest group (35 strains) was identified as *L. mesenteroides*, followed by *L. lactis* (27), *L. citreum* (24), *L. pseudomesenteroides* (13) and *W. paramesenteroides* (2; Facklam and Elliott, 1995). Reports on the association of *Weissella* spp. with bacteremia are extremely rare, and thus far, the involvement of *Weissella confusa* has been reported in only two cases (Green et al., 1990; Olano et al., 2001). In the latter case, *W. confusa* bacteremia was associated with polymicrobial infection of a 46-year-old male patient, and the presence of accompanying vancomycin-resistant bacteria was considered serious.

Intrinsic resistance to vancomycin in Gram-positive bacteria presumably predates acquired vancomycin resistance in enterococci, but it has only recently generated interest. Intrinsically resistant enterococci possessing the *vanC* gene and the nonenterococcal genera *Leuconostoc*, *Lactobacillus*, *Pediococcus* and *Erysipelothrix* are known to cause human infection. Available data on their identification, resistance mechanisms, epidemiology, clinical infections and antimicrobial susceptibility are examined in the review by Nelson (1999). Intrinsically vancomycin-resistant Gram-positive strains are usually opportunistic pathogens. Although serious infections may occur, treatment options remain available. No additional infection control measures for the intrinsically resistant genera appear justified with currently available evidence, although vigilance should be maintained to detect future changes in susceptibility patterns (Nelson, 1999). Golan et al. (2001) have shown daptomycin, a novel lipopeptide antibiotic, to be effective in the treatment of line-related *Leuconostoc* bacteremia and other infections with Gram-positive bacteria, including those resistant to vancomycin.

Continuing reports on the association of the genus *Leuconostoc* with clinical samples and human infections, support the earlier observations (Handwerger et al., 1990; Facklam and Elliott, 1995). Practically all patients involved either had been hospitalized owing to severe underlying diseases, had previous antibiotic therapy (especially vancomycin), and/or undergone procedures preventing normal host defense, or invasive procedures. Although the clinical significance of an infection with these bacteria in nonimmunocompromised patients is not clarified yet, Facklam and Elliott (1995) conclude that, considering the wide distribution of leuconostocs (and weissellas) in the environment and the few infections with which they have been associated, they have "very little virulence" for healthy human beings. It is the view of the authors (JB and WH) that there is no evidence or indication of real pathogenicity among any strains of *Leuconostoc* or *Weissella* studied thus far.

Practical Importance and Applications

Importance of Genus *Leuconostoc* in Meat Processing

As commercial starter organisms for meat fermentation, leuconostocs are not as important as

some *Lactobacillus* and *Pediococcus* spp., and particularly *Lactobacillus sakei* and *Lactobacillus curvatus* (cf. Holzapfel, 1998). However, the future use of these organisms in biopreservation remains an open issue. Meat leuconostocs produce bacteriocins (Hastings et al., 1994) that, if proved safe, may have practical applications. Parente et al. (2001) evaluated the evolution of LAB populations in traditional fermented sausages (salsiccia and soppressata) produced in artisanal and industrial plants in Basilicata, southern Italy. Out of 414 LAB strains isolated from samples of sausages at different stages of ripening, 7% were identified as *Leuconostoc* spp. (*L. carnosum*, *L. gelidum* and *L. pseudomesenteroides*). A bacteriocinogenic strain of *L. gelidum* (UAL 187) was effective in inhibiting a sulfide-producing *Lactobacillus sakei* strain in vacuum-packaged beef stored at 2°C (Leisner et al., 1996).

It would be important to obtain more data from the identification and characterization studies dealing with meat leuconostocs. Also, the spoilage potential of different *Leuconostoc* species and strains should be elucidated further. Studies have mainly focused on the identification of mixed flora components, but the true understanding of the spoilage potential of various strains is still unclear.

Leuconostoc and *Weissella* in Other Food Fermentations

DAIRY FERMENTATIONS In milk, leuconostocs grow in association with lactococci and may produce important flavor compounds. Particular strains therefore play a significant part in developing aroma and flavor in various cultured dairy products, including certain varieties of cheeses (Sandine and Elliker, 1970). The role of leuconostocs in various dairy applications and the factors that govern their significant functions has been reviewed by Vedamuthu (1994). Particular strains of *Leuconostoc* species are frequently used in mesophilic mixed strain cultures to produce aroma during milk fermentations. Strain 91404 of *L. mesenteroides* subsp. *cremoris* was selected by Levata-Jovanovic and Sandine (1997) as an aroma producer in the preparation of experimental cultured buttermilk on the basis of its low diacetyl reductase activity, citrate utilization, and high diacetyl production under acidic conditions, and also because of its growth characteristics and its compatibility with *Lactococcus* strains. Fortification of ripened buttermilk with sodium citrate resulted in a significant increase of diacetyl and acetoin production during buttermilk storage at 5°C for 2 weeks. Surplus of citrate, low pH of 4.5–4.7, a sufficient number of

active nongrowing aroma producers, air incorporation during curd breaking, and low temperature storage stimulated citrate metabolism and increased production and conservation of flavor during the 2 weeks of storage.

Optimal development of *L. mesenteroides* subsp. *cremoris* appears to be dependent on the manganese content of the milk, and with values <15 µg/liter, it may be outcompeted in a mixed strain starter culture. *Leuconostoc mesenteroides* subsp. *cremoris* plays an important role in the desired gas formation in the cheeses such as Gouda and Edam where it comprises ca. 5% of a typical starter culture, as compared to 2–3% for Tilsiter (Zickrick, 1996). It is known that leuconostocs play a minor role in "spontaneous" and most traditional milk fermentations. Beukes et al. (2001) collected 15 samples of conventionally fermented milk from households in South Africa and Namibia, and found the genera *Leuconostoc*, *Lactococcus* and *Lactobacillus* to predominate the microbial population, and identified 83% of the leuconostoc isolates as *L. mesenteroides* subsp. *dextranicum*, with *L. citreum* as a minor group.

VEGETABLE FERMENTATIONS *Leuconostoc* strains typically dominate the first fermentation stage of most plant food substrates. This has been shown for vegetable fermentations such as sauerkraut, cucumbers, olives and various low-salt pickle fermentations. Leuconostocs also seem to play the initiating part in palm wine fermentation (W. H. Holzapfel, unpublished observations) and in a number of traditional cereal fermentations, typical of various regions in Africa, and of Mexican pozol, a fermented maize dough (Ampe et al., 1999). In Sri Lanka and Southern India, leuconostocs dominate the "spontaneous" fermentation of legumes such as black dhal into idli and dosai. In addition to their contribution to leavening and souring, their association with these fermentations also seems to contribute to the degradation of antinutritive factors such as trypsin inhibitor and oligosaccharides such as raffinose and stachyose, causing flatulence (Shamala and Sreekantiah, 1988 and Holzapfel, 1998). Although most of these plant food fermentations are "spontaneous," the inclusion of *Leuconostoc* strains into starter cultures appears beneficial for the fermentation process and for the development of desirable sensory traits. Using a vegetable juice medium (VJM), Gardner et al. (2001) selected mixed starter cultures for lactic acid fermentation of carrot, cabbage, beet and onion vegetable mixtures. Greater differences were observed between the pure cultures as compared to mixtures with regard to growth in VJM and viability during storage. Reductions in viable cell counts during storage of the fer-

mented VJM occurred more rapidly with a *Leuconostoc* strain than for pediococci or lactobacilli. This selection process enabled the preparation of a mixed culture that was more rapid than the silage inoculants in acidifying the medium, and more effective in reducing the production of gas during fermentation and storage.

Ishikawa et al. (1999) used a psychrotolerant *Leuconostoc* strain (D-33), together with a strain of *Lactobacillus casei* (L-14) as starter culture for the saltless fermentation of radish pickles. In addition to the inhibition of undesirable bacteria, the sensory quality was improved during the 60 days of fermentation by 2,3-butanediol formation by the *Leuconostoc* strain, and by the prevention of browning. Out of 24 dairy *Leuconostoc* strains from French commercial starters, screened for biochemical and technical features. Following hierarchical clustering analysis, based on 58 morphological and biochemical characters, most strains fitted into three clusters at a similarity level of 65% and were identified as belonging to either *L. lactis* or *L. mesenteroides*. Fourteen strains selected on the basis of desirable technological properties were considered for designing of commercial starters (Server-Busson et al., 1999).

Genus *Oenococcus* in Food Fermentations

Oenococcus oeni is considered the most important and desirable species among the LAB involved in wine making. It plays a key role in the secondary fermentation or malolactic fermentation of wine, also as a result of their high resistance to SO₂ and ethanol. *Oenococcus oeni* plays a major role in the production of microbiologically stable and "mild" wines by converting L-malic acid to L(+) lactic acid (Davis et al., 1985a; Wibowo et al., 1985). Selected strains of *O. oeni* are used as starter cultures for improved control of the MLF, and are commercially available in the major wine growing areas of industrialized countries.

Herrero et al. (1999) conducted controlled malolactic fermentation of yeast fermented apple juice, using an *O. oeni* strain for improving the cider making process at the industrial level. *Oenococcus oeni* immobilized in alginate beads was applied as starter culture for controlled malolactic fermentation of cider. The rates of malic acid consumption were similar to conventional fermentation, but a lower ethanoic acid content and higher concentration of alcohols were detected with immobilized cells. These features were considered to have beneficial effects on the sensory properties of cider (Herrero et al., 2001). Nedovic et al. (2000) also succeeded in improving cider quality and to accelerate the process by continuous fermentation with co-immobilized yeast and *O. oeni* cells.

Genus *Leuconostoc* and *Weissella* in Non-food Fermentations

In an inoculation study, Cai et al. (1998a) observed that strains of *W. paramesenteroides* and *L. pseudomesenteroides* did not contribute to an improvement of silage quality and may rather cause fermentation loss, as compared to *Lactobacillus* strains.

The ability of *L. mesenteroides* subsp. *mesenteroides* to produce dextrans from sucrose by a dextransucrase has been exploited for the production of commercially valuable dextrans on an industrial scale and is mentioned elsewhere in this chapter. Several dextran-producing leuconostocs are able to produce different types of polymers such as alternans, dextrans and levans (Cote and Ahlgren, 1995). To develop strategies for improved dextransucrase production, Dols et al. (1997) studied dextran production in relation to the growth and energetics of *L. mesenteroides* NRRL B-1299 during metabolism of various sugars. For sucrose-grown cultures, they found that a large fraction of sucrose is converted outside the cell by dextransucrase into dextran and fructose without supporting growth. The fraction entering the cell is phosphorylated by an inducible sucrose phosphorylase and converted to glucose-6-phosphate (G-6-P) by a constitutive phosphoglucomutase and to heterofermentative metabolites (lactate, acetate and ethanol). Sucrose was found to support a higher growth rate (0.98 h⁻¹) than the monosaccharides.

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CHAPTER 1.2.10

The Genera *Lactobacillus* and *Carnobacterium*

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Lactobacilli are Gram-positive, nonsporeforming rods, catalase-negative when growing without a heme source (e.g., blood), usually nonmotile, and occasionally nitrate reducers. They utilize glucose fermentatively (Kandler and Weiss, 1986a) and may be either homofermentative, producing more than 85% lactic acid from glucose, or heterofermentative, producing lactic acid, CO_2, ethanol, and/or acetic acid in equimolar amounts. The type species is *Lactobacillus delbrueckii* Leichmann 1896 (Beijerinck, 1901). The genus *Lactobacillus* constitutes together with the genus *Pediococcus* the family Lactobacillaceae and presently comprises 80 recognized species and 15 subspecies. The phylogenetic position of this family in relation to lactic acid bacteria (LAB) and closely related genera is depicted in Fig. 1. The increased interest in microbial ecology and the availability of sensitive taxonomic methods delivering unambiguous identification results have created a marked increase in the number of species within the genera *Lactobacillus* and *Carnobacterium* since the previous edition of *The Prokaryotes*. The validly described species of the genus *Lactobacillus* are listed in Table 1, and those of the genus *Carnobacterium* are treated under a separate headline. *Lactobacillus rogosae* is not included in Table 1 as no type strain with the original description is available in culture collections (Kandler and Weiss, 1986a). In addition, *L. catenaformis* and *L. vitulinus* have not been included because 16S rRNA sequence analyses show that these species are closely related to *Clostridium ramosum*, *C. spiroforme*, *C. cocleatum* and the genus *Coprobacillus*.

In Table 1, the numbers in column I indicate species for which 16S rRNA sequence data of 90% of the bases are available. These have been used to construct phylogenetic trees shown in Figs. 2–9. The species attribution to phylogenetic groups (shown in Figs. 3–9) as well as to groups of main habitats is included in Table 1. On the basis of the results of genotypic studies, the genus *Lactobacillus* comprises a defined group of organisms, which consists of subgroups (Table 1, column V) that are sufficiently genotypically distinct as to justify their attribution to several genera. The recent creation of the genus *Weissella* from a homogenous group of heterofermentative LAB (formerly included in the genus *Lactobacillus*; Collins et al., 1993) is a consequent step in that direction, and the description of *Paralactobacillus* follows this development. On the other hand, the morphological, biochemical and physiological characteristics of the lactobacilli are usually not so diverse that they demand a separation into different genera.

The genus *Carnobacterium* had also been included in the genus *Lactobacillus* (Collins et al., 1987). It presently contains 7 validly described species, with *Carnobacterium divergens* being the type species. These organisms share some habitats with lactobacilli but differ in certain physiological properties, the most important of which are listed in Table 2. One species, *C. piscicola*, contains strains pathogenic to fish. Thus, the separation of *Carnobacterium* from *Lactobacillus* renders the lactobacilli a homogeneous group of nonpathogenic bacteria. However, one exception has recently been described. *Lactobacillus psittaci* was isolated from an inflamed air sac of a dead parrot. Nothing is known about the infectiveness, virulence factors, or the natural habitat of this species. In general, lactobacilli are useful to humans in several respects: They are indispensable agents of the fermentation of foods and feed, and are constituents of the human (as well as animal) body flora, wherein they exert health promoting effects. On the other hand, together with certain *Carnobacterium* species, they are involved in food spoilage. On the basis of the taxonomic history of the genera *Lactobacillus* and *Carnobacterium*, common physiological properties, and their common occurrence in certain habitats, treatment of the two genera within one chapter is justified. In the first edition of *The Prokaryotes*, the genus *Lactobacillus* was excellently treated by M. E. Sharpe (Sharpe, 1981). New results and developments especially of ecological, practical, taxonomical interest were added to the second edition, and the process of updating the knowledge is continued in this chapter.
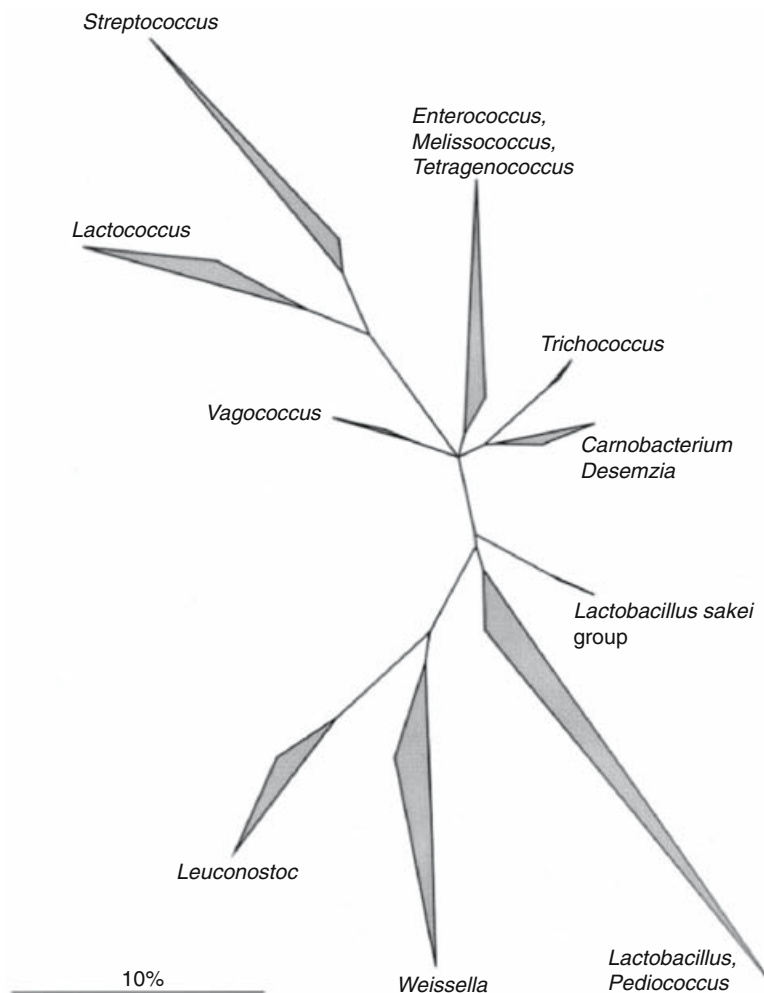
Fig. 1. Phylogenetic tree depicting the position of Lactobacillaceae and carnobacteria in relation to lactic acid bacteria (LAB) and closely related genera. The consensus tree is based on maximum parsimony analyses of all available, at least 90% complete 16S rRNA sequences of Gram-positive bacteria. The topology was evaluated and corrected according to the results of distance matrix and maximum likelihood analyses with various data sets. Alignment positions that share identical residues in at least 50% of all sequences of the depicted genera were considered. Multifurcations indicate that a common branching order could not be significantly determined or was not supported, when performing different alternative treeing approaches. The bar indicates 10% estimated sequence divergence.

The Genus Lactobacillus

Habitat

Lactobacilli are strictly fermentative and have complex, sometimes very fastidious nutritional requirements for carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives, and vitamins. Large amounts of lactic acid and small amounts of other compounds are the products of their carbohydrate metabolism. Lactobacilli grow in a variety of habitats, wherever high levels of soluble carbohydrate, protein breakdown products, vitamins, and a low oxygen tension occur. They are aciduric or acidophilic, different species having adapted themselves to grow under widely different environmental conditions, and their production of high levels of lactic acid lowers the pH of the substrate and suppresses the growth of many other bacteria; these factors account for the wide distribution of lactobacilli and their successful establishment in many markedly different habitats (Sharpe, 1981). The occurrence of the various species in the main groups of habitats is shown in Table 1.

Humans and Animals

ORAL CAVITY The oral cavity of humans and animals contains several types of epithelial surfaces and is the only site that contains hard non-

Table 1. List of the species of the genus *Lactobacillus*.

I[a]	II[b]	III[c]	IV[d]	V[e]
1[f]	*L. acetotolerans* Entani et al. 1986	B	D	de
2	*L. acidipiscis* Tanasupawat et al. 2000	B	F	sl
3[f]	*L. acidophilus* (Moro 1900) Hansen and Mocquot 1970	A	I	de
4[f]	*L. agilis* Weiss et al. 1981	B	S	sl
5[f]	*L. algidus* Kato et al. 2000	B	D	sl
6[f]	*L. alimentarius* (Reuter 1970) Reuter 1983	B	F, D	pl
7[f]	*L. amylolyticus* Bohak et al. 1998	A	F	de
8[f]	*L. amylophilus* Nakamura and Crowell 1979	A	F	de
9[f]	*L. amylovorus* Nakamura 1981	A	F	de
10[f]	*L. animalis* Dent and Williams 1982	A	I	sl
11[f]	*L. arizonensis* Swezey et al. 2000	B	F	pl
12a[f]	*L. aviarius* subsp. *aviarius* Fujisawa et al. 1984	A	I	sl
12b	*L. aviarius* subsp. *araffinosus* Fujisawa et al. 1984	A	I	sl
13[f]	*L. bifermentans* (Pette and van Beynum 1943) Kandler et al. 1983a	B	D	u
14[f]	*L. brevis* (Orla-Jensen 1919) Bergey et al. 1934	C	F, D	u
15[f]	*L. buchneri* (Henneberg 1903) Bergey et al. 1923	C	F, D	bu
16[f]	*L. casei* (Orla-Jensen 1916) Hansen and Lessel 1971	B	I, F, D	ca
17[f]	*L. coleohominis* Nikolaitchouk et al. 2001	C	I	re
18[f]	*L. collinoides* Carr and Davies 1972	C	D	pl
19a[f]	*L. coryniformis* subsp. *coryniformis* Abo-Elnaga and Kandler 1965	B	F	u
19b	*L. coryniformis* subsp. *torquens* Abo-Elnaga and Kandler 1965	B	F	u
20[f]	*L. crispatus* (Brygoo and Aladame 1953) Cato et al. 1983	A	I	de
21a[f]	*L. curvatus* subsp. *curvatus* (Troili-Petersson 1903) Abo-Elnaga and Kandler 1965	B	I, F, D	sa
21b	*L. curvatus* subsp. *melibiosus* Torriani et al. 1996	B	F, D	sa
22[f]	*L. cypricasei* Lawson et al. 2001a	B	F	sl
23a[f]	*L. delbrueckii* subsp. *delbrueckii* (Leichmann 1896) Beijerinck 1901	A	F	de
23b[f]	*L. delbrueckii* subsp. *bulgaricus* (Orla-Jensen 1919) Weiss et al. 1983	A	F	de
23c[f]	*L. delbrueckii* subsp. *lactis* (Orla-Jensen 1919) Weiss et al. 1983	A	F	de
24[f]	*L. diolivorans* Krooneman et al. 2002	C	F	bu
25[f]	*L. durianis* Leisner et al. 2002	C	F	re
26[f]	*L. equi* Morotomi et al. 2002	A	I	sl
27[f]	*L. farciminis* (Reuter 1970) Reuter 1983	A	F	pl
28[f]	*L. ferintoshensis* Simpson et al. 2001	C	F	bu
29[f]	*L. fermentum* Beijerinck 1901	C	F, D	re
30[f]	*L. fornicalis* Dicks et al. 2000	B	I	de
31[f]	*L. fructivorans* Charlton et al. 1934	C	D	bu
32[f]	*L. frumenti* Müller et al. 2000a	C	F	re
33	*L. fuchuensis* Sakala et al. 2002	B	D	sa
34[f]	*L. gallinarum* Fujisawa et al. 1992	A	I	de
35[f]	*L. gasseri* Lauer and Kandler 1980	A	I	de
36[f]	*L. graminis* Beck et al. 1988	B	F	sa
37[f]	*L. hamsteri* Mitsuoka and Fujisawa 1987	B	I	de
38[f]	*L. helveticus* (Orla-Jensen 1919) Bergey et al. 1925	A	F	de
39[f]	*L. hilgardii* Douglas and Cruess 1936	C	D	bu
40[f]	*L. homohiochii* Kitahara et al. 1957	B	D	bu
41[f]	*L. iners* Falsen et al. 1999	A	I	de
42[f]	*L. intestinalis* (Hemme 1974) Fujisawa et al. 1990	B	I	de
43[f]	*L. jensenii* Gasser et al. 1970	B	I	de
44[f]	*L. johnsonii* Fujisawa et al. 1992	A	I	de
45[f]	*L. kefiranofaciens* Fujisawa et al. 1988	A	F	de
46[f]	*L. kefirgranum* Takizawa et al. 1994	A	F	de
47[f]	*L. kefiri* corrig. Kandler and Kunath 1983	C	F	bu
48[f]	*L. kimchii* Yoon et al. 2000	B	F	pl
49[f]	*L. kunkeei* Edwards et al. 1998	C	D	bu
50[f]	*L. lindneri* (Henneberg 1901) Back et al. 1996	C	D	bu
51[f]	*L. malefermentans* (Russell and Walker 1953) Farrow et al. 1988	C	D	pl
52[f]	*L. mali* Carr and Davies 1970, emend. Kaneuchi et al. 1988	A	D	sl
53[f]	*L. manihotivorans* Morlon-Guyot et al. 1998	A	F	ca
54[f]	*L. mucosae* Roos et al. 2000	C	I, F	re
55[f]	*L. murinus* Hemme et al. 1980	B	I	sl
56	*L. nagelii* Edwards et al. 2000	A	D	sl
57[f]	*L. oris* Farrow and Collins 1988	C	I	re
58[f]	*L. panis* Wiese et al. 1996	C	F	re

Table 1. *Continued*

Iª	IIᵇ	IIIᶜ	IVᵈ	Vᵉ
59ᶠ	*L. pantheris* Liu and Dong 2002	A	I	ca
60	*L. parabuchneri* Farrow et al. 1988	C	I	bu
61aᶠ	*L. paracasei* subsp. *paracasei* Collins et al. 1989	B	I, D, F	ca
61bᶠ	*L. paracasei* subsp. *tolerans* (Abo-Elnaga and Kandler 1965) Collins et al. 1989	B	D	ca
62ᶠ	*L. parakefiri* corrig. Takizawa et al. 1994	C	F	bu
63ᶠ	*L. paralimentarius* Cai et al. 1999	B	F	pl
64ᶠ	*L. paraplantarum* Curk et al. 1996	B	D, I	pl
65ᶠ	*L. pentosus* (Fred et al. 1921) Zanoni et al. 1987	B	F, S	pl
66ᶠ	*L. perolens* Back et al. 1999	B	D	u
67ᶠ	*L. plantarum* (Orla-Jensen 1919) Bergey et al. 1923	B	F, D	pl
68ᶠ	*L. pontis* Vogel et al. 1994	C	F	re
69ᶠ	*L. psittaci* Lawson et al. 2001b	A	(P)	de
70ᶠ	*L. reuteri* Kandler et al. 1980	C	I, F	re
71ᶠ	*L. rhamnosus* (Hansen 1968) Collins et al. 1989	B	F	ca
72ᶠ	*L. ruminis* Sharpe et al. 1973a	A	I	sl
73aᶠ	*L. sakei* subsp. *sakei* corrig. Katagiri et al. 1934	B	I, F, D	sa
73b	*L. sakei* subsp. *carnosus* corrig. Torriani et al. 1996	B	F, D	sa
74aᶠ	*L. salivarius* subsp. *salivarius* Rogosa et al. 1953	A	I	sl
74bᶠ	*L. salivarius* subsp. *salicinius* Rogosa et al. 1953	A	I	sl
75ᶠ	*L. sanfranciscensis* corrig. (Kline and Sugihara 1971) Weiss and Schillinger 1984	C	F	bu
76ᶠ	*L. sharpeae* Weiss et al. 1981	A	S	ca
77ᶠ	*L. suebicus* Kleynmans et al. 1989	C	F	re
78ᶠ	*L. vaccinostercus* Okada et al. 1979	C	I	re
79ᶠ	*L. vaginalis* Embley et al. 1989	C	I	re
80ᶠ	*L. zeae* (Kuznetsov 1959) Dicks et al. 1996	B	F	ca

Abbreviations: A, obligately homofermentative; B, facultatively heterofermentative; C, obligately heterofermentative; D, food associated, usually involved in spoilage; F, involved in fermentation of food and feed; I, associated with humans and/or animals, e.g., oral cavity, intestines, vagina; S, sewage; (P), opportunistic pathogen; bu, *L. buchneri* group; ca, *L. casei* group; de, *L. delbrueckii* group; *Pl*, *L. plantarum* group; re, *L. reuteri* group; sa, *L. sakei* group; sl, *L. salivarius* group; and u, unique.
ªIn column I, numbers have been assigned to species to facilitate their identification in Tables 5, 6 and 7.
ᵇColumn II provides the full taxon name of each species.
ᶜColumn III is the type of glucose fermentation of the species.
ᵈColumn IV is the main habitat of the species.
ᵉColumn V is the phylogenetic group as determined by C. Hertel (unpublished results).
ᶠSpecies for which at least 90% of the complete 16S rDNA sequences have been published. These species were considered for construction of the phylogenetic trees (Figs. 2 and 3). Partial sequences have been considered to species to phylogenetic related groups.

Table 2. Key characteristics for differentiating lactobacilli from carnobacteria.

Characteristic	*Lactobacillus*	*Carnobacterium*
Growth on actetate agar (pH 5.4)	+	−
Growth at pH 4.5	+	−
Growth at pH 9.0ª	−	+
Lactic acid isomers produced	L(+), D(−), DL	L(+)
Type of diamino acid in peptidoglycanᵇ	Lys, mDpm, Ornᵇ	mDpmᵇ
Fermentation type (glucose)	Homo or hetero	Atypical homoᶜ
G+C content (mol%)	32–55	33.0–37.2

Symbols and abbreviations: +, present; −, absent; mDpm, *meso*-diaminopimelic acid; homo, predominant fermentation product is lactic acid; and hetero, CO_2, ethanol, and acetic acid are produced in addition to lactic acid.
ªIn D-MRS-broth.
ᵇAbbreviations based on Schleifer and Kandler (1972).
ᶜGlycolysis with minor and retarded CO_2 production (De Bruyn et al., 1988).

shedding surfaces (on teeth) for microbial colonization (Jenkinson, 1999). It is estimated that more then 500 bacterial species inhabit the oral cavity (Moore and Moore, 1994), which is an open system that is constantly exposed to exogenous factors, especially during breathing and the intake of food. The composition and density of the microbial population differs in the various localities and is determined mainly by three ecological determinants: nutrition, redox potential, and adhesion. In addition, numerous additional factors are operative (Knoke and Bernhardt, 1986; Jenkinson, 1999; Slots and Chen, 1999), including 1) genetics, affecting for example the saliva (flow rate, redox potential, buffer capacity and pH, and concentration of ions, salts and enzymes), gender, and hormonal status, 2) the swallowing process, 3) the diet (composition, consistency, and frequency of meals), 4) microbial interactions, 5) mouth hygiene, 6) health status, and 7) treatment with antimicrobials.

The anatomical features, in combination with the previously mentioned factors, will determine the relative proportions of the bacterial groups. In these groups, LAB and especially oral streptococci are of superior importance for health status. In general, lactobacilli have been found to constitute <0.1% of either the cheek or tongue bacteria, <0.005% of intragingival plaque, and <1% each of the saliva and the gingival crevice bacteria (Marsh and Martin, 1984). Studies of tooth surfaces of infants and young children (Carlsson and Gothefors, 1975) have shown that lactobacilli are present only in very small numbers or as transients in the mouth. Lactobacilli developing in the mouth of 2- to 5-year-old children consisted mostly of *L. casei* and *L. rhamnosus*, and occasionally of *L. acidophilus* and *L. fermentum*. *Lactobacillus casei* was found especially in children with carious lesions. Rogosa et al. (1953), in their classic paper on the identification of oral lactobacilli, identified 500 strains isolated from saliva specimens of 130 school children. *Lactobacillus casei* and *L. fermentum* were the predominant species present in 59 and 45% of the samples, respectively, while *L. acidophilus* and *L. brevis* in 22 and 17% of the samples, respectively, were also present. *Lactobacillus buchneri*, *L. salivarius*, *L. plantarum* and *L. cellobiosus* occurred less frequently. Those findings have been confirmed by other studies on children and adults (London, 1976) that found a similar distribution of species, and *L. casei* and *L. fermentum* were found to predominate. All later investigations confirm clearly that the closely related *L. casei-L. rhamnosus* are by far the most important lactobacilli on the basis of both general presence in individuals and numbers. The heterofermentative *L. fermentum* (*L. cellobiosus*) is commonly ranked second. Owing

to changes in nomenclature, it should be noted that the isolated *L. casei* are probably *L. paracasei* (Collins et al., 1989), and *L. cellobiosus* are *L. fermentum* (Vescovo et al., 1979). Heterofermentative *Lactobacillus* strains isolated from human saliva and classified as *L. brevis* (Hayward and Davis, 1956; Hayward, 1957) were found not to be genetically or biochemically related to this or other recognized heterofermentative species, and were classified into a new species, *L. oris* (Farrow and Collins, 1988a).

All strains of lactobacilli isolated from deep dental plaque by Shovell and Gillis (1972) were *L. casei*, and other work has confirmed that this species is the prevalent *Lactobacillus* in plaque (Basson and Van Wyk, 1982; Depaola, 1989; Hahn et al., 1989; Wijeyeweera and Kleinberg, 1989). Kneist et al. (1988) found that *L. rhamnosus* was the dominant *Lactobacillus* in softened and hard carious dentin of 125 deciduous molars. In relative importance, this species was followed by *L. plantarum*, *L. casei* subsp. *casei*, *L. curvatus*, *L. xylosus* and *L. coryniformis*. In a more recent study of lactobacilli associated with active caries lesions (Botha et al., 1998), their occurrence in the dentin of 12 patients with open caries (group A) was compared with that in the saliva of 12 individuals (group B) with no caries (DMFT = 0). Of the 153 isolates differentiated on the basis of phenotypical characteristics, homofermentative species (group A = 82% and B = 90%) were predominantly *L. paracasei* (group A = 39% and B = 30%) and *L. rhamnosus* (group A = 31% and B = 41%), heterofermentative species (group A = 18% and B = 10%) were predominantly *L. fermentum* (group A = 68% and B = 100%), and the remainder in group A were *Weissella confusa* and *W. minor*. In addition in group A, the authors found 16% *L. murinus*, and at lower percentage (summing up to 14%), *L. hamsteri*, *L. casei*, *L. graminis*, *L. acidophilus*, *L. jensenii* and *L. sakei*. In group B, 29% of the homofermentative association constituted *L. murinus*, *L. hamsteri*, *L. acidophilus*, *L. graminis*, *L. jensenii*, *L. sakei*, *L. homohiochii*, *L. curvatus*, *L. pentosus* and *L. salivarius*. It is remarkable that species such as *L. murinus*, *L. hamsteri* and *L. graminis* (commonly considered to be associated with mice, hamsters and grass, respectively) had been identified. Furthermore, *L. salivarius* was found at a rather low incidence.

Although present in carious lesions, the lactobacilli are not considered to be actively involved in caries progression. Kneist et al. (1988) and Russel and Ahmed (1978) have shown that neither *L. acidophilus* nor *L. casei* or *L. fermentum* is able to form plaque alone (i.e., without the participation of *Streptococcus mutans* or *S. sanguis*). Yet, lactobacilli were found at the site of 85% of progressive lesions before clinical diag-

nosis of caries progression was made (Boyar and Bowden, 1985). Using a modified Rogosa SL agar medium (with melezitose as the only sugar), Claesson and Crossner (1985) found *L. casei* to be the most common oral *Lactobacillus* species, representing a higher proportion of the salivary *Lactobacillus* population in children than in adults. It is recognized that the microbial population of the saliva contains microorganisms from different areas of the mouth, and although it has been suggested that dental plaque contributes little to this population (Hardie and Bowden, 1974; Hakgudener, 1985; Matee et al., 1985), several reports indicate a definite relationship between the incidence of caries and the *Lactobacillus* population of the saliva (Minah et al., 1985; Krasse, 1988; Alaluusua et al., 1989; Bjarnason, 1989). Owing to fluctuations in numbers of lactobacilli and pH of the saliva, Sullivan and Schroeder (1989) concluded that lactobacilli had only low caries-predictive ability for children from 5 to 7 years of age. Data from other workers, however, contradicted this report, and specifically suggest the salivary *Lactobacillus* population to be a useful tool for early diagnosis of caries (Crossner and Unell, 1986; Vanderas, 1986; Wikner, 1986; Krasse, 1988). Neither the use of fluoride (gel) toothpaste, fluoride-containing mouth rinse (Brown et al., 1983; Etemadzeh et al., 1989), nor fluoride-containing chewing gum (Ekstrand et al., 1985), or even chlorhexidine treatments (Lundstrom and Krasse, 1987) was found to decrease oral lactobacilli numbers significantly. By contrast, *S. mutans* numbers were reduced by chlorhexidine treatment (Lundstrom and Krasse, 1987).

Detailed studies of dental plaque show that the pioneer bacteria (first binders) to a preformed pellicle are streptococci, *Neisseria*, *Actinomyces* and *Capnocytophaga* (Jenkinson, 1999). Other bacteria coadhere to that community and increase the complexity of the association. Dental caries is caused by the metabolism of this association, which attacks the tooth enamel. Caries is thus most probably initiated by the combined activities of several species. There is strong evidence that the progression of the disease is promoted by streptococci of the *S. mutans* group. At that stage also, lactobacilli come into play. Only at that stage may lactobacilli multiply within the built up matrix. The frequent consumption of fermentable carbohydrates increases the production of lactic acid and changes the ecological conditions in the niche, and it follows a selection for more acidophilic bacteria among which lactobacilli, and *L. paracasei*/*L. rhamnosus* in particular, are of primary importance. Nothing is known about a specific role of the multitude of *Lactobacillus* species

that occur in significant but minor numbers. The effect of carbohydrates on the selective growth of LAB, and above all lactobacilli, at the expense of less acid tolerant species, is the basis of the "ecological plaque hypothesis" (Marsh, 1994). It includes, that the low pH (initiated by the streptococci) found in carious cavities favors lactobacilli. The high count may at least in part be the result of caries and not the cause (Hardie and Bowden, 1974; Alaluusua et al., 1987; Wijeyeweera and Kleinberg, 1989).

INTESTINAL TRACT A recent comprehensive treatment of gastrointestinal (GI) microbiology including aspects of anatomy, ecology, modeling, taxonomy, and host interactions can be found in a monograph edited by Mackie et al. (1997a); (1997b). The GI tract of vertebrates harbors a large and complex association of microbes. Most knowledge of the intestinal microbial association has been obtained from analysis of feces inasmuch as access to the intestines is, at least in healthy humans, extremely difficult, and even sampling by application of automatic capsule systems does not deliver results that are representative of a defined section at a defined time. Human feces contain $>10^{11}$ bacteria per gram, and microbial cells make up about 55% of the solids within the colon (Tannock, 1995). More then 400 bacterial species can be isolated from one subject, with obligate anaerobe bacteria being predominant and exceeding in number facultative anaerobes by 100–1000 times (Moore and Holdemann, 1974). The bacterial numbers and composition vary considerably between different animal species and along the GI tract (the colon being the most highly colonized) owing to anatomical and physiological distinctions. In vertebrate animals and humans, the intestinal microflora is similarly complex, but the ecological importance of their lactobacilli differs. *Lactobacillus* species comprise only a minor part of the bacterial community in human feces (Mitsuoka, 1992; Sghir et al., 2000), whereas animals such as pigs, chicken, dogs, mice, rats and hamsters harbor greater *Lactobacillus* populations in their intestines (Mitsuoka, 1992). Additionally, the *Lactobacillus* species composition in feces and intestinal contents vary between the different hosts (Table 3). The role of lactobacilli in intestinal ecosystems has received much attention, especially with respect to their beneficial effect on human and animal health, e.g., when ingested as probiotics.

The Ecosystem The GI tract is an open ecosystem in contact with the environment. Therefore, lactobacilli isolated from gut contents or feces do not necessarily inhabit this ecosystem but may be

Table 3. *Lactobacillus* species in intestines of humans and animals.

Species	Human	Pig	Chicken	Cattle	Dog	Mouse	Rat	Hamster	Horse	Jaguar*
L. acidophilus	+	+	+	+	nd	+	+	nd	nd	nd
L. amylovorus	nd	M	nd	+	nd	nd	nd	nd	+	nd
L. crispatus	M	+	M	nd	nd	nd	nd	nd	+	nd
L. gallinarum	nd	nd	M	nd	nd	nd	nd	nd	nd	nd
L. gasseri	M	nd	nd	+	nd	+	nd	nd	nd	nd
L. johnsonii	+	+	M	M	nd	nd	nd	nd	+	nd
L. murinus/animalis	nd	nd	+	nd	M	M	+	nd	nd	nd
L. intestinalis	nd	nd	nd	nd	nd	M	M	nd	nd	nd
L. salivarius	M	M	M	nd	nd	nd	nd	nd	M	nd
L. agilis	nd	+	+	nd	nd	nd	nd	nd	+	nd
L. ruminis	M	nd	nd	M	nd	nd	nd	nd	nd	nd
L. hamsteri	nd	nd	nd	nd	nd	nd	nd	M	nd	nd
L. aviarius	nd	nd	+	nd	nd	nd	nd	nd	nd	nd
L. paracasei	+	nd	nd	+	nd	nd	nd	nd	nd	nd
L. rhamnosus	+	nd	nd	nd	nd	nd	nd	nd	nd	nd
L. plantarum	+	+	nd	+	nd	nd	nd	nd	+	nd
L. reuteri	M	M	M	M	M	M	M	M	M	nd
L. fermentum	+	+	+	+	nd	+	+	nd	nd	nd
L. brevis	+	+	+	+	nd	nd	nd	nd	nd	nd
L. delbrueckii	+	nd	nd	nd	nd	+	+	nd	nd	nd
L. sakei	+	nd	nd	nd	nd	nd	nd	nd	nd	nd
L. mucosae	nd	+	nd	nd	nd	nd	nd	nd	nd	nd
L. equi	nd	nd	nd	nd	nd	nd	nd	nd	M	nd
L. pantheris	nd	nd	nd	nd	nd	nd	nd	nd	nd	+

Symbols: M, major component of *Lactobacillus* species; +, occasionally recovered; *, the species composition has not been studied so far; and nd, no data.
From Marounek et al. (1988); Mitsuoka (1992); Stewart (1992); Sarra et al. (1992); Tannock (1992); Rubio et al. (1998); Gusils et al. (1999); Roos et al. (2000); Tannock et al. (2000); Walter et al. (2000, 2001); Yuki et al. (2000); Liu and Dong (2002); Morotomi et al. (2002); and Dal Bello et al. (2003).

transient or allochthonous. In the human intestine, they may originate from fermented food, the oral cavity, and food of plant origin or stored meat products. From these sources they are continuously ingested in high numbers. Similarly in animals, lactobacilli may originate from their feed.

The main ecological parameters affecting microbial growth in the mammalian fermentative gut compartments are 1) a pH ranging from 5.5 to 6.9 (with lower values in proximal and higher values in the distal colon), 2) anaerobiosis (redox potential ranging from –350 to –400 mV), 3) temperature (37–41°C), and 4) osmolality (250–350 mmol/kg; Mackie, 1997a). In addition to vertical differences in the ecological conditions within the GI tract, those in the lumen and in the mucus layer must also be considered. Mucus consists of glycoproteins (secreted as mucin by specialized goblet cells) and sloughed epithelial cells. It forms a layer of approximately 400 µm (Wold, 1999) covering the epithelial cells, and provides a continuous substrate supply (2–3 g/day) for bacteria. Close to the mucosa, the oxygen tension is relatively high, favoring the growth of facultative anaerobes, which in their turn contribute to the strict anaerobicity of the lumen.

Little is still known of the controlling effect on intestinal growth of bacteria caused by so-called "defensins." These antagonistic peptides are secreted together with lysozyme and other hydrolytic enzymes by Paneth cells in Leberkühn's crypts, which are most abundant in the small intestine (reviewed by Axelsson and Mahida, 2000). Defensins are cationic, arginine-rich peptides consisting of 28–44 amino acids, are synthesized as pre-propeptides (Boman, 1998; Ayabe et al., 2000). They resemble bacteriocins in their mode of action. In the large intestine, which contains the main mass of fermentatively active bacteria, normally few Paneth cells are found. However, these can be upregulated in the large intestine in response to inflammation or neoplasia. Factors such as defensins together with specific bacterial adherence conditions and immunotolerance phenomena may determine the composition of an individual's intestinal microbial flora. Lactobacilli therein require for their fermentative metabolism carbohydrates, preferentially simple monosaccharides up to oligosaccharides. Unlike these simple carbohy-

drates, undigested carbohydrate sources (starch, plant cell wall polysaccharides, and oligosaccharides) reach the large intestine in substantial amounts. Cassidy et al. (1994) estimated daily supply of carbohydrates as follows: resistant starch, 8–40 g; non-starch polysaccharides (e.g., cellulose, hemicellulose, pectin, and inulin), 8–18 g; not absorbable sugars and sugar alcohols, 2–10 g; and chitin and amino sugars, 1–2 g. These carbohydrates are mainly not or poorly fermented by lactobacilli, which may therefore depend on carbohydrates derived from the hydrolytic activity of more potent bacteria, such as bifidobacteria, *Bacteroides* spp., etc. On the other hand, Hartemink (1999) showed that intestinal lactobacilli can ferment certain undigestible carbohydrates, often considered as prebiotics (see below), such as galacto- and fructo-oligosaccharides, palatinose, lactitol, raffinose and stachyose. In addition, starch hydrolysis is not uncommon in strains of *L. acidophilus* group. As the supply of nitrogen sources (Cassidy et al., 1994) and growth factors is not limited, the large intestine can be a suitable environment for lactobacilli.

Lactobacilli in Humans Lactobacillus species can be isolated from feces of human subjects at counts varying greatly from none to $< 10^9$ colony-forming units (cfu)/g feces (Mitsuoka, 1992; Kimura et al., 1997; Tannock et al., 2000). Present knowledge indicates that 14 species are associated with the human gut (Table 3), but only certain species and especially strains thereof can be isolated from individuals over longer periods. Studies conducted between 1960 and 1980 indicated that *L. acidophilus*, *L. fermentum*, *L. salivarius* and an anaerobic lactic acid bacterium, previously named "*Catenabacterium catenaforme*" are the dominant autochthonous *Lactobacillus* species (Lerche and Reuter, 1961; Reuter, 1965a; Mitsuoka, 1969; Moore and Holdeman, 1974; Mitsuoka et al., 1975). On the basis of current taxonomy, most of the *L. acidophilus* isolates nowadays are classified as *L. gasseri* and *L. crispatus*, and most of the *L. fermentum* strains belong to *L. reuteri* (Mitsuoka, 1992; Reuter, 2001). Isolates identified as *Catenabacterium catenaforme* were later identified as nonmotile variants of *L. ruminis* (Reuter, 2001). In a more recent study, Tannock et al. (2000) studied the succession of lactobacilli in feces of 10 human subjects and concluded that *L. ruminis* and *L. salivarius* are autochthonous, since they could recover defined strains of these species for at least 18 months. *Lactobacillus ruminis* was also detected by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) as the predominant species over several

months, whereas detection of other *Lactobacillus* species fluctuated (Walter et al., 2001; Heilig et al., 2002). These studies indicated that lactobacilli such as *L. paracasei*, *L. rhamnosus*, *L. delbrueckii*, *L. brevis*, *L. plantarum* and *L. fermentum* are rather transient, persist for limited times, or in undetectably low numbers that may increase in response to dietary factors or changes in the host's condition. This conclusion is in accordance with findings of Bunte et al. (2000), Jacobsen et al. (1999), and Reuter (1965a) showing that some food-associated lactobacilli survive the passage through the intestine. Other food-associated lactobacilli, especially *L. sakei* and *L. curvatus*, could be detected by direct analyses of 16S rRNA genes using specific primers but not by bacteriological culture on Rogosa SL agar (Walter et al., 2001; Heilig et al., 2002). Alternative cultivation methods, including incubation on acetate-free media at 30°C in a 2% O_2 atmosphere showed that strains of *L. sakei* (and numerous additional food-associated lactobacilli) are viable and can be cultured from human fecal samples (Dal Bello et al., 2003).

Our knowledge of the intestinal flora in sections of the intestinal tract rests on the study of samples obtained using an automatic capsule system or from post mortem cases. It was shown the *Lactobacillus* species could be detected in all parts of the human intestine including the stomach, which is characterized by a pH of around 3.0 (2.2–4.2). Relatively few bacterial species can tolerate these acidic conditions and most organisms ingested with food and saliva are killed by the hydrochloric acid, reducing the population to about 10^3 cfu/ml containing mainly lactobacilli and streptococci (enterococci; Reuter, 1965a; Drasar and Hill, 1974). The flora of the small intestine increases from $<10^4$ bacteria per ml of digesta in the duodenum up to 10^8–10^9 bacteria per gram of feces in the terminal ileum (Gorbach et al., 1967; Drasar and Hill, 1974; Tannock, 1995; Reuter, 2001). In the duodenum and jejunum, lactobacilli and enterococci are the dominant bacteria (Mitsuoka, 1992; Reuter, 2001). The flora becomes more complex in the ileum, being qualitatively similar to that of the large intestine, and the relative proportion of lactobacilli drops. Samples from the proximal and distal parts of the colon as well as from feces show a rather similar bacterial composition, with lactobacilli being more numerous in the colon, especially in the proximal section, than in feces (Reuter, 1965b; Marteau et al., 2001).

Lactobacilli in Animals In contrast to humans, the proximal portions of the digestive tracts of pig, mouse and rat harbor large populations of bacteria (about 10^8 bacteria per gram of con-

tent). Unlike the human stomach, which is lined with a glandular mucosa, the stomach of pigs, mice and rats is lined partly with a non-glandular, squamous stratified epithelium (Tannock, 1992). These regions, the "pars oesophagea" of pigs and the forestomach of mice and rats, are colonized by lactobacilli (forming a layer of bacterial cells) adhering directly to the epithelium. Lactobacilli shed from this layer inoculate continuously the digesta, and lactobacilli are therefore detected in large numbers throughout the gastrointestinal tract (Tannock, 1997b). Cell counts for lactobacilli in the rodent forestomach, colon and cecum can exceed numbers of 10^8 bacteria per gram of content with slightly lower counts in the duodenum (10^6–10^7), jejunum and the ileum (10^7–10^8). Direct analysis of 16S rRNA genes from the content of ilea and large intestines of pigs showed that *L. amylovorus*, *L. johnsonii* and *L. reuteri* are numerically dominant (4.5, 3.2 and 2.1% of all clones, respectively; Leser et al., 2002). The application of these culture independent molecular techniques revealed that common food-associated lactobacilli (in particular *L. pontis* and *L. panis*) are present in the gut of pigs. These species had hitherto not been found by bacteriological culture (Simpson et al., 2000; Leser et al., 2002). A new species, *L. pantheris*, was detected in the feces of jaguars (Liu and Dong, 2002).

Lactobacilli mainly colonize squamous, stratified epithelium present in the crop of many types of birds and in the stomach of horses as a dense layer of bacteria, *L. salivarius* being the predominant organism in the crop of birds (Mead, 1997). In addition, *L. aviarius* with the subspecies *aviarius* and *araffinosus* were isolated from the feces and alimentary tract of chickens and ducks (Fujisawa et al., 1984). Adhering microorganisms were isolated from the nonsecreting area of the horse stomach and identified as *L. salivarius*, *L. crispatus*, *L. reuteri* and *L. agilis* (Yuki et al., 2000). *Lactobacillus equi* was not recovered at that time but occurred in the feces of horses.

The mechanism by which *Lactobacillus* strains adhere to these epithelia has not yet been determined, but preliminary in vitro investigations have shown that both carbohydrate and protein molecules are involved (Tannock, 1997b). *Lactobacillus* strains that adhere to epithelial cells show specificity for an animal host. Strains originating from the rodent forestomach do not adhere to crop cells, while isolates from poultry do not adhere to epithelial cells from the rodent forestomach and the pars oesophagea of pigs (Tannock, 1997b). However, some exceptions occur. For example, *Lactobacillus* strains isolated from chicken adhered to pig squamous epithelial cells (Tannock et al., 1982) and a strain of

L. reuteri, isolated from calf feces, adhered markedly to the squamous epithelium of the mouse stomach (Sherman and Savage, 1986).

Relatively little is still known about the *Lactobacillus* population of the ruminant and especially of the rumen of adult animals. The numbers of lactobacilli vary according to the age and diet of the animal. In adult sheep and cattle, LAB constitute usually only a minor component of the microbial flora of the rumen (Stewart, 1992). They are, however, predominant in the ruminal populations of young animals as well as in animals being fed or being switched to high-grain, starch diets (Hespell et al., 1997). Under these conditions, the rumen pH can drop to 4.5, and counts of lactobacilli and *Streptococcus bovis* can reach >10^9cfu/ml. At that very low rumen pH, the growth of other rumen bacteria, including the lactate utilizers, is suppressed (Stewart, 1992). The excessive accumulation of lactic acid in the rumen leads to a condition known as "lactic acidosis," which is accompanied with loss of appetite and, in the most severe cases, death of the animal (Stewart, 1992). Many (but not all) LAB are sensitive to animal feed antibiotics, and it had been suggested that some of these compounds my help to prevent the development of lactic acidosis (Stewart, 1992).

Although insects may play a role as vectors for the dissemination of lactobacilli, little is known about the lactobacilli in these animals. Kvasnikov, Kotljar, and Vasileva (cited by London, 1976) isolated strains of *L. casei* and *L. cellobiosus* from the honeybee, silkworm moth, and from fruit flies; Ruiz-Argueso and Rodriguez-Navarro (1975) found *L. viridescens* (*Weissella*) in the stomach of the honeybee. Shrivastava (1982) suggested a new species, *Lactobacillus eurydice*, to be typically associated with the honeybees and bumblebees; this species name however has not been validated. In the midgut and rectum of honeybees, 10^8–10^9 anaerobic Gram-positive acidoresistant rods were found (Rada et al., 1997). The favorable effect of *L. acidophilus* and *L. bulgaricus*, administered in combination with propionibacteria via mulberry leaves to silkworms was reported by Rizvanov et al. (1982). Also in the foreguts of dog chow-fed cockroaches an abundant population of LAB was observed, which were not identified to species level. It was estimated that acetate and lactate produced in the foregut of cockroaches could support up to 14% of the insects' respiratory requirement if taken up and used by the animal (Kane and Breszak, 1991). In a study of 44 *Lactobacillus* strains isolated from the intestine of insects (mainly termites), Tina (1987) grouped 31 strains as homofermentative, and identified 10 as *L. brevis* and 3 as *L. cellobiosus*.

Role of Adherence in Colonization When attached and replicating on a host's surface, a microbe species can persist in a flowing habitat whereas nonadherent microbes are transported away by the flow of secretion (Tannock, 1995). Therefore, bacterial adherence to intestinal epithelia could play a role in colonization of the human gut or persistence in this ecosystem after ingestion as a probiotic (Vaughan et al., 1999). It has been shown that some lactobacilli have the ability to bind to intestinal mucus and surface structures of the mucosa and this trait might be involved in intestinal colonization (Roos and Jonsson, 2002). Lactobacilli adhere to the human intestinal cell lines Caco-2 and HT-29 (Haller et al., 2001) or enterocytes harvested from the intestinal tract (Tannock, 1997a). Comparative studies with the wildtype and a nonaggregating mutant of *L. crispatus* showed the mutant was less adherent to Caco-2 cells and to mucus and was not recovered from human intestinal contents (Cesena et al., 2001). On the other hand, members of the normal microflora colonize mucus associated with tissue surfaces to only a very limited extent in humans, and the numbers of bacteria obtained from washed tissue surfaces are considerably lower than those observed in studies of rodents (Tannock, 1995). Evidence for significant association of lactobacilli with the columnar epithelium of the human gastrointestinal tract is inconclusive and has not been observed in vivo (Tannock, 1999a). Furthermore, in vitro tests showed that adherence is highly dependent on certain physiological conditions (e.g., pH conditions not found in the gut), and no correlation was observed between the in vivo findings and adherence of a certain bacterial strain to epithelial cells or cell lines. Thus it appears impossible to predict the success of a strain as a colonizer of the human gut (Wold, 1999; Morelli, 2000). The knowledge of the natural colonization pattern of lactobacilli in the human gut is still rudimentary and more research is necessary to confirm the role of adherence in colonization or persistence.

The Acquisition of Lactobacilli The fetus of vertebrates exists in a sterile environment until birth and becomes rapidly colonized by bacteria after nativity. In humans, lactobacilli can be cultured from the feces (10^5–10^6 cells per gram) of some newborns 1–3 days after birth (Sakata et al., 1985; Benno and Mitsuoka, 1986). From weeks 1–19, varying numbers of lactobacilli (around 10^7 cells per gram of feces) can be detected in ca. 50% of all human infants (Conway, 1997). Since the early work of Tissier (1905), it has been suggested that *L. acidophilus* (*Bacillus acidophilus*) is a predominant organism in bottle-fed infants,

whereas bifidobacteria predominate in breast fed infants (Mitsuoka, 1992). Benno et al. (1984) found higher numbers of lactobacilli in bottle-fed compared with breast-fed infants. Later studies did not show a difference between the two groups of infants (Adlerberth, 1999). These variable results can at least partially be explained by differences in the composition of the formulae used in the various studies. As described by Lönerdal (1999) and Gil and Rueda (2000), the concentration of compounds such as iron, nucleotides, and fatty acids as well as the whole composition of the diet can affect the composition of the intestinal flora. However, little is known of the specific effect on the *Lactobacillus* association. The mode of delivery had a significant influence on early colonization and infants born by cesarean delivery had a delayed colonization with lactobacilli (Gronlund et al., 1999). Low birth weight infants also had a delayed emergence of *Lactobacillus* species compared with normal weight infants (Sakata et al., 1985). The fecal flora (including lactobacilli) of weaned children closely resembles that of adults. In elderly persons, marked alterations can be observed with significantly higher numbers of lactobacilli but less bifidobacteria in feces (Mitsuoka and Hayakawa, 1973).

Lactobacilli are among the pioneer organisms that colonize the gastrointestinal tract of pigs, mice and rats and can be detected in appreciable numbers 24 h after birth (Tannock, 1992). It has been shown that strains colonizing the pars oesophagea of piglets early in life were replaced by other *Lactobacillus* strains as time progressed. From days 7–14 after birth, one *Lactobacillus* strain predominated suggesting that a bacteriologically stable situation had developed (Tannock et al., 1990). The *Lactobacillus* strains predominating in the contents of the piglet rectum differed from those predominating in the gastric region of the host.

Effect of Lactobacilli on the Host The normal microflora of the digestive tract has marked influences on the animal host (Tannock, 1995). Comparisons between animals harboring a normal microflora (conventional) and germ-free animals have shown that the bacteria modify the biochemistry, physiology and immunology (Tannock, 1997a). The intestinal flora influence the host's health, including nutrition, physiological function, drug efficacy, carcinogenesis, aging as well as the hosts immunological responses, resistance to infection, and responses to endotoxin and various other stresses (Mitsuoka, 1992). Microbial species that are likely candidates responsible for particular activities can be suggested in some cases, but in general there is a

paucity of information on the role of specific microbial components of the microbiota in the ecosystem (Tannock, 1997a).

An important function of the indigenous intestinal microbiota is its ability to interfere with colonization of the intestinal tract by exogenous microorganisms, including pathogens (Rolfe, 1997). Terms such as bacterial antagonism, competitive exclusion, bacterial interference, and colonization resistance have been used to describe this function. One possible mechanism by which the indigenous microflora may inhibit the establishment of pathogenic microorganisms is by influencing the peristalsis of the intestine. Lactobacilli dominate the small intestine of humans and most mammals and may increase the rate at which the digesta is propelled by peristaltic movement through the small bowel (Tannock, 1997a). This difference, which has been observed by comparing conventional with germ-free rodents, is probably due the production of lactic acid by the lactobacilli. Lactic acid, which is not detectable in the gastrointestinal tract of germ-free rats, was able to stimulate intestinal motility in vitro (Yokokura et al., 1977b). The desirable predominance of lactobacilli in the small intestine helps to prevent the potentially lethal diarrhea or scouring that occurs in young animals when enteropathogenic coliforms proliferate in the upper GI tract. For example, the presence of lactobacilli in the small intestine of newborn piglets results in an acid intestinal environment that is inhibitory to *Escherichia coli* and *Vibrio cholerae* enterotoxin production and may protect newborn pigs against these microorganisms (Rolfe, 1997).

Similarly, a decrease of pH in the large intestines can exert a marked effect on the ecology and the composition of the microbial flora. The concept of prebiotics aims to achieve such a shift by adding to the diet carbohydrates that are not digested in the small intestines. These become substrates for LAB (mainly bifidobacteria), which through their fermentative metabolism achieve the desired decrease of pH (Hammes and Dal Bello, 2002). Prebiotics are defined as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improve host health" (Gibson and Roberfroid, 1995). To some extent the composition of the intestinal lactobacilli are also affected by consumption of prebiotics such as fructo-oligosaccharides and galacto-oligosaccharides (see above).

The Role of Bacteriocins and Other Antagonistically Active Compounds Apart from lowering the pH of the gut contents, antimicrobial substances (e.g., lactic acid, hydrogen peroxide, and bacteriocins; Jack et al., 1995) produced by intestinal lactobacilli may also contribute to this protective effect (Tannock, 1992). Probably all species of lactobacilli include strains producing bacteriocins, such as lactocin B (Barefoot and Klaenhammer, 1983), lactacin F (Muriana and Klaenhammer, 1991), acidocin A (Kanatani et al., 1995) produced by *L. acidophilus*, acidocin B (Leer et al., 1995) by *L. johnsonii*, reutericin 6 by *L. reuteri* (Kabuki et al., 1997), and ABP-118 by *L. salivarius* ssp. *salivarius* (Flynn et al., 2002). Additionally, non-bacteriocin (often called "BLIS" [bacteriocin-like inhibitor substances]) and noncharacterized antibacterial activities have been described for *Lactobacillus* strains, which often inhibit enteropathogenic bacteria including *Salmonella typhimurium*, *Shigella flexneri*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Listeria monocytogenes* (Bernet-Camard et al., 1997; Coconnier et al., 1997; Gäzle et al., 2000) and protozoa like *Gardia intestinalis* in vitro (Perez et al., 2001). Reuterin, produced by *L. reuteri* in the presence of glycerol, also inhibits a great range of bacteria and additionally protozoa and fungi (Talarico and Dobrogosz, 1989). The ecological significance of antibacterial substances in the intestine is still inconclusive and it should be considered that what happens in the test tube does not necessarily predict an antibacterial effect in the intestinal tract. For example, it has been demonstrated that susceptibility of lactobacilli or enterococci to the bacteriocin lactacin F, assayed on agar plates, could not be confirmed in vivo (Tannock, 1999a). Furthermore, a missing quorum-sensing signal (e.g., cell counts that are too low in the intestines) may prevent gene expression (Swift et al., 2000). Some strains of lactobacilli were antagonistic to the growth of pathogens in the intestinal tract (mainly in mouse models with *Salmonella typhimurium*) and the cause of these effects was assumed to be at least partly due to inhibitory substances (Bernet-Camard et al., 1997; Coconnier et al., 1997; Hudault et al., 1997). The ecological significance of these antimicrobial compounds has not been confirmed by comparing non-BLIS-producing isogenic mutants with corresponding BLIS-producing wildtypes and, therefore, the true antagonistic principle was not conclusively identified.

The Role of Bile Salt Hydrolases Bile salt hydrolases catalyze the hydrolysis of conjugated bile acids, which enter the small bowel in bile and are important for the emulsification, digestion and absorption of dietary lipid present in the proximal small bowel (Tannock, 1998). Deconjugated bile acids are much less efficient and can

damage the epithelium of the upper small bowel. This becomes evident in patients suffering from "contaminated small bowel syndrome," a dysbiosis in which "fecal type" bacteria with bile salt hydrolase activity colonize the upper region of the small bowel and exert a deleterious effect on the host (Tannock, 1995). In the case of mice, where lactobacilli are numerous in the upper intestinal tract and responsible for almost all bile salt hydrolase activity, the decrease in the concentration of conjugated bile acids in the small bowel does not appear to exert a negative effect, i.e., the growth rate of the animals is not affected whether or not they are colonized by lactobacilli and regardless of how much bile salt hydrolase the lactobacilli produce (Bateup et al., 1995).

Most intestinal isolates of lactobacilli and some lactobacilli involved in food fermentations exhibit bile salt hydrolase activity (Haller et al., 2001; Moser and Savage, 2001). The genes encoding bile salt hydrolases have been characterized for *L. johnsonii* 100-100, *L. acidophilus* KS-13 and *L. plantarum* 80 (Christiaens et al., 1992; Elkins et al., 2001). Whether producing bile salt hydrolase favors lactobacilli colonization of the intestinal tract is not readily apparent. The bile acid nucleus is not degraded by lactobacilli, and taurine is not incorporated into bacterial cell proteins (Tannock, 1998). It has been suggested that deconjugation of bile acids is a detoxification mechanism and protects the cells from the deleterious effect of conjugated bile salts (De Smet et al, 1995; De Boever and Verstraete, 1999). However, recent findings did not support this hypothesis and showed that hydrolase activity did not correlate with resistance (Moser and Savage, 2001). Additionally, deconjugated bile salts are more toxic than their conjugated counterparts (especially at low pH), and it has been shown that bile or bile salts are more toxic for wildtype strains compared to their bile salt hydrolase negative mutants (De Boever and Verstraete, 1999; Grill et al. 2000). Bile salt hydrolase activity is suggested to be important at some level for lactobacilli to colonize the human intestine (Moser and Savage, 2001). In contrast, strains of lactobacilli having different amounts of bile salt hydrolase activity were able to colonize the gastrointestinal tract of mice equally well (Bateup et al., 1995).

Studies with Reconstituted Lactobacillus-free Mice Animals lacking just one of the normal complement of bacterial microflora provide a good experimental model for studying microbial influences on the host (Tannock, 1992). Mice with gastrointestinal tract microflora lacking lactobacilli but otherwise identical (tested by comparing twenty-six microflora-associated characteristics) with conventional animals were used to study the influence of lactobacilli on the host and other members of the gut flora (Tannock et al., 1988). Studies using these reconstituted *Lactobacillus*-free mice (RLF mice) have shown the following: 1) The numbers of Enterobacteriaceae in the digestive tract of adult mice is the same with or without lactobacilli (Tannock et al., 1988); 2) the composition of the large bowel microflora is the same whether lactobacilli are present or absent (Tannock et al., 1988); 3) azoreductase activity was 31% lower in the cecum of mice colonized by lactobacilli (McConnell and Tannock, 1991); 4) male RLF mice had about 52% more cecal β-glucuronidase activity than did their female counterparts, and colonization of male mice by lactobacilli reduced the β-glucuronidase activity to the level of female mice (McConnell and Tannock, 1993); 5) the presence of lactobacilli as members of the digestive tract microbiota did not influence the total concentration of cholesterol or the amount associated with the high density lipoprotein fraction in the serum (Tannock, 1997b); 6) bile salt hydrolase activity detected in RLF mice was reduced by 86% in the distal small bowel (74% in the cecum) compared to RLF animals whose gastrointestinal tract was colonized by lactobacilli (RLFL mice), and a comparison of RLFL mice with conventional mice revealed that lactobacilli are the main contributors to total bile salt hydrolase activity in the murine intestinal tract (Tannock et al., 1989); and 7) the major portion of the bile acids in the small bowel contents of RLFL mice was deconjugated (67.9%) in contrast to RLF animals, where a smaller portion of the bile acids was deconjugated (23.5%), demonstrating that bile salt hydrolase produced by lactobacilli was active under the conditions prevailing in the proximal bowel of mice (Tannock et al., 1994).

Lactobacilli and Health The presence of the lactic microflora, and especially lactobacilli, in the digestive tract has historically been considered as beneficial to the host. At the beginning of the last century, Elie Metchnikoff (1845–1916) stated that toxic substances produced by members of the intestinal microflora are absorbed from the intestinal tract and contribute to the aging process (Tannock, 1995). Microbes capable of degrading proteins (putrefaction), releasing ammonia, amines and indole were considered harmful, and bacteria like lactobacilli (which ferment carbohydrates to obtain energy and have little proteolytic activity) were thought to be beneficial. The extent to which lactobacilli colonizing the intestine contribute to the health of a healthy human is still hypothetical. Ecological studies revealed that only a minority of human subjects contained true autochthonous *Lactoba-*

cillus strains and some did not contain any culturable lactobacilli (Tannock et al., 2000; Walter et al., 2001). It has never been reported that these subjects are less healthy or more susceptible to infections.

Metchnikoff supposed that the number of putrefactive microbes in the intestine could be replaced or diminished by enriching the normal microflora with LAB. This supposition was based on the observation that these bacteria prevented the putrefactive spoilage of milk, and that eastern European peasants, some of whom were apparently long-lived, consumed fermented dairy products as part of their diet. There is conflicting experimental data on the ability of LAB present in these products (*Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus helveticus*) to survive the passage through the intestinal tract (Pochart et al., 1989; Pedrosa et al., 1995; Jacobsen et al., 1999). However, other LAB ingested with fermented food were found to survive in the intestinal tract in high numbers (Fernandes et al., 1992; Jacobsen et al., 1999; Bunte et al., 2000). Additionally, lactobacilli isolated from feces have been used more recently in the preparation of probiotic dairy products intended to promote health. Oral probiotics are referred to as living microorganisms which (upon ingestion in certain numbers) exert health benefits beyond inherent basic nutrition (Guarner and Schaafsma, 1998). Metchnikoff's concept to implant LAB in the intestine proved to be realistic but only for short periods. Strains ingested with fermented food or probiotics could colonize the human gut only transiently and disappeared from the feces of most subjects within a couple of days (Jacobsen et al., 1999; Tannock et al., 2000; Bezkorovainy, 2001). It has been reported that even without a permanent colonization, lactobacilli benefit the health of consumers as follows:

1) Dairy products containing LAB, including lactobacilli, are better tolerated by persons with lactose intolerance (Fernandes et al., 1992; Saavedra, 2001), since the bacterial β-galactosidase supports the hydrolysis of lactose in the small intestine.

2) Preparations containing lactobacilli were effective in the treatment of diarrhea of various etiology (Fernandes et al., 1992; Saavedra, 2001; Van Niel et al., 2002), whereas the results of prevention of diarrhea studies are contradictory (Saavedra, 2001; Szajewkda and Mrukowicz, 2001).

3) Lactobacilli exert effects on the immune system of the host (Isolauri et al., 2001; Perdigon et al., 2001). These effects include modulation of cytokine gene expression, stimulation of phagocytosis by peripheral blood leucocytes, and an increase of serum IgA and IgM titers (Schiffrin et al., 1995; McCracken and Gaskins, 1999; Haller et al., 2000). The physiological significance of this immunomodulation has not conclusively been shown in all cases. Products containing lactobacilli exhibit anti-carcinogenic properties (Fernandes et al., 1992; Hirayama and Rafter, 1999) and they had been successfully used to treat and prevent atopic disease and infections (Cross et al., 2001; Hatakka et al., 2001). These effects are thought to be mediated, at least partly, through a lactobacillus-induced enhancement of the immune response.

4) Lactobacilli reduce pro-carcinogenic enzymes in feces and have the potential to bind and degrade carcinogens (Fernandes et al., 1992; Hirayama and Rafter, 1999).

5) Although studies of probiotics in therapy of intestinal bowl disease (IBD) are at an early stage, encouraging data have already been obtained in experimental murine models, including IL-10 knock-out mice. In this model, lactobacilli were shown to reduce mucosal inflammation (Madsen et al., 1999). At an age of two weeks, these mice displayed changes in bacterial colonization characterized by increased adherent and translocated bacteria as well as reduced numbers of lactobacilli. The rectal administration of *L. reuteri* enhanced the mucosal barrier function and attenuated the development of the colitis at an age of four weeks. Similar effects were observed in mutant mice upon daily administration of *L. plantarum* (Schultz et al., 2002). The established inflammation could be reversed and the onset of colitis prevented upon a pretreatment of gnotobiotic mutant mice. Human studies have been performed under placebo-controlled, double-blind conditions with patients suffering from chronic pouchitis (Gionchetti et al., 2000). The probiotic contained in the so-called "VSL-3" (consisting of *L. casei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii* spp. *bulgaricus*, bifidobacteria and *Streptococcus thermophilus*) was highly effective in maintenance of remission in the patients.

Many of the effects attributed to the ingestion of lactobacilli and other LAB including probiotics, however, remain convoluted and scientifically unsubstantiated, and it is rare that specific health claims can be made (Sanders, 1993; Tannock, 1999b). Lactobacilli as part of a traditional human diet or probiotic therapy may influence the homeostasis between the intestinal microflora and the host, but their mode of action in the prevention or even treatment of certain disease remains to be clarified. Furthermore, it has been shown that effects are strain specific, and only a limited number of *Lactobacillus* strains are investigated thoroughly (Reid,

1999a). More research is necessary to confirm the influence of ingested lactobacilli on the health of the consumer and to understand the mechanisms lying behind these effects.

HUMAN VAGINA In the healthy adult woman, the pH of the vagina throughout the menstrual cycle ranges between 3.5 and 4.5 (Redondo-López et al., 1990). The *Lactobacillus* species predominant in the vaginas of normal women are believed to maintain this low pH through their fermentative activities and to protect against invasion of undesirable microorganisms (Hill et al., 1985). On the other hand, the vaginal pH frequently does not correlate with the presence of lactobacilli and the vaginal secretions are already acidic at birth despite sterility of the vagina. Therefore the contribution of lactobacilli to the low pH is not yet fully understood (Redondo-López et al., 1990; Boskey et al., 1999). Their numbers determined by vaginal washings vary between 3.7 and 9.8 $\log_{10}$/ml (Redondo-López et al., 1990). Classically, glycogen is regarded as the only source of fermentable carbohydrate present in the vagina. However, Rogosa and Sharpe (1960) found that only some of their isolates fermented glycogen, and Wylie and Henderson (1969) found only 3 of their 42 isolates were positive. Stewart-Tull's finding (Stewart-Tull, 1964) that vaginal strains of *L. acidophilus* could ferment glycogen only in the presence of normal human serum (containing a glycogenase) suggested that the majority of vaginal lactobacilli are likely to obtain available carbohydrate from enzymatic breakdown of the polysaccharide by tissues or possibly by other organisms.

The history of studies of the vaginal microflora started with Döderlein (1894), and Döderlein's bacilli are now classified as lactobacilli. Rogosa and Sharpe (1960), who cite the earlier literature, identified isolates from normal, nonpregnant women as *L. acidophilus* (67%), *L. fermentum* (19%), *L. casei* subsp. *rhamnosus* (10%), and *L. cellobiosus* (4%). Other studies relying on phenotypical testing confirmed the predominance of *L. acidophilus* and *L. fermentum* and reported the occasional isolation of *L. casei*, *L. plantarum*, *L. brevis*, *L. delbrueckii*, *L. lactis*, *L. bulgaricus*, *L. leichmanii* and *L. salivarius* (Lenzner, 1966; Wylie and Henderson, 1969). New species were subsequently detected for which no other habitat but the female urogenital tract is known. *Lactobacillus vaginalis* has been isolated from the vagina of patients suffering from trichomoniasis (Embley et al., 1989). *Lactobacillus fornicalis* was isolated from the posterior fornix of healthy patients attending pre- and postnatal clinics (Dicks et al., 2000) and *L. iners* from the urine and vaginal discharge from adult women (Falsen et al., 1999). Remarkably, *L. iners* does not grow

on Man, Rogosa and Sharpe (MRS) or Rogosa agar but needs culturing on blood agar. Finally, *L. coleohominis* was isolated from the vaginas of two young, healthy women (Nikolaitchouk et al., 2001).

When isolates were identified with methods targeting the genotype, a more homogenous vaginal *Lactobacillus* association can be observed. Using DNA-DNA hybridization technique, Giorgi et al. (1987) did not detect *L. acidophilus* in the vaginal secretions of 27 asymptomic women but instead found *L. crispatus*, *L. jensenii*, *L. fermentum* and *L. gasseri* as the predominant species. In a recent investigation of 200 isolates from 23 healthy Swedish women (Vásquez et al., 2002), the isolates from blood agar, MRS- and Rogosa-agar were analyzed by combined use of random amplified polymorphic DNA (RAPD), temperature gradient gel electrophoresis (TGGE), and 16S rDNA sequencing. It was found that the vaginal flora of most subjects was dominated by a single RAPD type, and five of them harbored two types representing two different species or strains. The most frequently detected species were *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*. A greater variation of species with low probability occurrence was revealed by whole chromosomal DNA probe analysis of the vaginal lactobacilli isolated from 215 sexually active women (Antonio et al., 1999). Detected species (listed in decreasing order of occurrence) included *L. crispatus* (32%), *L. jensenii* (23%), an unknown species (15%), *L. gasseri* (5%), *L. fermentum* (0.3%), *L. oris* (0.3%), *L. reuteri* (0.3%), *L. ruminis* (0.3%) and *L. vaginalis* (0.3%). Thus, more recent studies show that an association of mainly *L. crispatus*, *L. gasseri*, *L. jensenii* and probably *L. iners* is predominant, while species such as *L. rhamnosus*, *L. paracasei*, *L. fermentum* and *L. plantarum* occur with less probability. The results of earlier work may reflect the unreliability of phenotypical taxonomic methods (especially when applied to different species of the *L. acidophilus* group), differences in geographical regions, the way samples are taken and treated, as well as the vaginal status (Vásquez et al., 2002).

Bacterial vaginosis (BV), a mild infection of the lower female genital tract (Reid and Heinemann, 1999b), is commonly associated with the presence of a variety of Gram-negative rod-shaped bacteria, such as *Fusobacterium*, *Prevotella*, *Peptostreptococcus*, *Porphyromonas*, *Mobiluncus* and *Mycoplasma* species (Spiegel et al., 1980; Mardh and Taylor-Robinson, 1984; Van der Meijden, 1984; Westrom et al., 1984; Cook et al., 1989; Hillier et al., 1993; Holst et al., 1994). It is unclear by which mechanism these bacteria displace or replace the normal *Lactobacillus* association. In addition, BV can lead to

complications in pregnancy, premature birth, death of the newborn, as well as acquisition of sexually transmitted disease (Gibbs et al., 1992; McGregor et al., 1993; Chaim et al., 1997; Goldenberg et al., 1997; Newton et al., 1997; Sewankambo et al., 1997). The therapy of BV starts usually with antibiotic treatment to remove the pathogen. A restoration of the "normal *Lactobacillus*-association" is the aim of the application of probiotics (Reid and Heinemann, 1999b; McLean and Rosenstein, 2000). There is indication that suitable bacterial strains with colonization potential should possess properties such as formation of the antagonistic compounds hydrogen peroxide (Klebanoff et al., 1991; Hillier et al., 1992; Song et al., 1999) and/or bacteriocin, adherence to the vaginal mucosa (Boris et al., 1998), production of surfactant (Velraeds et al., 1996), coaggregation ability with uropathogens (Reid et al., 1988), and resistance to spermicidal agents.

LACTOBACILLI AND PATHOGENICITY   The ingestion of large numbers of diverse lactobacilli together with contaminated and in particular fermented food as well as their presence in high numbers in the human body flora justifies the assumption that this group of microorganisms is safe for humans. On the other hand, lactobacilli have been isolated from diseased persons, thus indicating that once the organisms have gained access to the aseptic part of the body, they can survive, multiply and might become involved in diseases. The cases of infections by lactobacilli are very rare and have been estimated to be 0.05–0.48% of all cases of infective endocarditis and bacteremias (Gasser, 1994; Saxelin et al., 1996; Husni et al., 1997). In most of the rare cases, an underlying disease indicated a predisposition of the patients. Severe dental infections or recent dental manipulations were identified for 75% of cases of endocarditis as the main portal of entry for the lactobacilli (Sussman et al., 1986). Husni et al. (1997) reviewed the cases of 45 patients with clinically significant *Lactobacillus* bacteremia. It was reported that underlying conditions were common, including cancer (40%), recent surgery (38%), and diabetes mellitus (27%). Twenty-two patients were in the intensive care unit at the onset of bacteremia. Eleven were receiving immunosuppressive therapy, 11 parenteral nutrition, and 23 had received antibiotics without activity against lactobacilli prior to the occurrence of bacteremia. The bacteremia was polymicrobial in 27 patients and developed in 39 during hospitalization. Thirty-one patients died, but only one death was attributable to *Lactobacillus* bacteremia.

Three main groups of infections can be differentiated: endocarditis, bloodstream infections and local infections, which have been reviewed by Aguirre and Collins (1993) and Gasser (1994). The most frequently isolated *Lactobacillus* species were *L. rhamnosus*, *L. paracasei* and *L. plantarum*. Additional species are listed in Table 4, which shows the importance of these species for food fermentation including their use as probiotics. The inclusion of *L. casei* into the list takes into account the ongoing discussion of the nomenclature of the "*L. casei*" group (see below). To our knowledge, the isolation of *L. casei* (Orla-Jensen) Hansen and Lessel 1971, from patients has not been reported. Reported isolation of *L. casei* before the description of *L. paracasei* and *L. rhamnosus* by Collins et al. (1989) might have been a misclassification. From Table 4, it can be concluded that the isolates may originate from intestines and/or food. This observation attracted much attention in the evaluation of probiotic food safety. The probiotic organisms contained therein are usually isolates from feces often belonging to *L. paracasei* or *L. rhamnosus*, and two cases of human infection by *L. rhamnosus* were traced to possible probiotic consumption (Rautio et al., 1999; Mackay et al., 1999). The safety of these LAB in food has been discussed by Borriello et al. (2003).

Properties that determine pathogenicity of lactobacilli are unknown. As for the safety of intestinal lactobacilli in food little is known, their application needs to consider ceratin aspects. Clearly, the formation of biogenic amines in food is undesirable, and excessive formation of D-lactate may be harmful for short bowel patients. The D-lactate producing lactobacilli may overgrow commensal bacteria and cause D-lactic acidosis (Coronado et al., 1995;

Table 4. Ecology of *Lactobacillus* species that might have been involved in human infections.

| Species | Main habitat | | |
	Human body	Fermented food	Probiotic food
L. acidophilus	+[a]	+	+
L. brevis	+	+	−
L. casei	−	+	−
L. delbrueckii	+	+	+
L. fermentum	+[a]	+	−
L. gasseri	+	−	−
L. jensenii	+	−	−
L. johnsonii	+	+	+
L. paracasei	+	+	+
L. plantarum	+	+	+
L. rhamnosus	+	+	+
L. salivarius	+	−	−

Symbols: +, present; and −, absent.
[a]The early species identification may be doubtful (see also section Intestinal Tract—Lactobacilli in Humans).

Bongaerts et al., 1997). In intestinal flora, properties considered as disadvantageous in food bacteria have become criteria in the evaluation procedure. Examples of such properties are binding to extracellular matrices (particularly to collagen, or serum proteins fibrinogen and fibronectin) and aggregation of human platelets (Harty et al., 1994). Furthermore, deconjugation of bile salts and degradation of mucin need consideration (BgVV, 1999). Harty et al. (1994) studied the relevant properties of 5 strains of *L. paracasei* and 5 of *L. rhamnosus* isolated from infective endocarditis (IE) patients. The IE-strains were compared with 10 laboratory strains of the same species and also with various oral isolates. With *L. rhamnosus*, it was observed that platelets aggregated with all IE isolates and with half of the reference strains. With *L. paracasei*, 2 out of 5 IE isolates and 2 out of 9 reference strains reacted positively. Platelet aggregation was furthermore a property of oral strains of *L. acidophilus* (1/1), *L. salivarius* (2/3), *L. plantarum* (3/5) and *L. fermentum* (2/3). Thus, platelet aggregation is dispersedly spread among lactobacilli and not restricted to isolates from diseased persons. The authors included in their studies the potential to bind to the plasma proteins (fibrinogen and fibronectin) as well as to collagens. The results showed the properties had a dispersed distribution as was observed for platelet aggregation. The results were also in agreement with those of a preceding report (Harty et al., 1993), which revealed just a significant tendency of the IE isolates to exhibit higher hydrophobicity, hydroxyapatite adhesion, and salivary aggregation values than observed for laboratory strains. Thus, the results confirm that no specific properties related to pathogenicity can be attributed to lactobacilli isolated from diseased persons.

Plants and Materials of Plant Origin

In the plant phyllosphere of rye, wheat, sugar beet and olive, 85 species of microorganisms in 37 genera have been detected by culture-based methods (Thompson et al., 1993; Legard et al., 1994; Hirano and Upper, 2000). The majority of the bacteria are commensals with no apparent effect on the plant. With the availability of culture independent methods, it became evident that the multitude of microorganisms is even greater. Using denaturing gradient gel electrophoresis (DGGE) to study the microbial phyllosphere populations of 7 plant species, Yang et al. (2001) have observed that the community structures were similar on different individuals of the same species, but unique on different plant species. Of the bacteria represented on the gel by 7 unique sequences, only 4 could be attributed to

known phyllosphere bacteria. Among the species identified by DGGE, no lactic acid bacteria (LAB) were found. As DGGE analysis usually detects not more than 90–99% of the main species in a population, it can be concluded that these bacteria play only a minor role in the phyllosphere. This conclusion is consistent with the estimation of Daeschel et al. (1987) that LAB represent 0.01–1% of the total phyllosphere population. Large variations in the LAB count have been reported to occur on leaves, with numbers ranging from below detectability to $<10^8$ cfu/g. These variations are caused by the effects of factors such as climate, harvest season, ultraviolet (UV)-light, location on the plant, exposure to dust, fertilization, mechanical stresses, or lesions caused by insects. In addition, it was shown by Müller and Seyfarth (1997) that LAB on grass occur as "somnicells" (Roszak and Colwell, 1987) and need resuscitation to be recovered. By incubating the hand cut grasses in yeast extract (5 g/liter) or peptone plus vitamins, the LAB counts increased after 8 h of incubation and within the following 20 h went from below detectable levels ($<10^1$ cfu/g) to values up to ca. 10^8 cfu/g. This result may also partially explain the so-called "apparent chopper inoculation" phenomenon (Pahlow et al., 1995), which consists of the observation that the microbial counts of grasses (including maize) are dramatically higher upon harvest with machines than those determined for hand cut grass from the same area. It is suggested the mechanical forces at machine harvest release sap from the cells which supports the resuscitation of the LAB. Otherwise, LAB remain in a VBNC (viable but not culturable) state, as they find usually harsh conditions in the phyllosphere, especially because nutrients are not readily available on the intact epidermis. This aspect was investigated by Leveau and Lindow (2001), who found 0.15–4.6 pg of fructose as the local intial concentrations on the leaves of *Phaseolus vulgaris*. For their studies, the authors inoculated the leaves with a reporter construct of *Erwinia herbicola* harboring a green fluorescence gene under the control of the *fruB* promoter of *E. coli*.

The location of microbial populations on leaves of 9 plant species has been studied by Morris et al. (1997). With the aid of epifluorescence microscopy, scanning electron microscopy, and confocal laser microscopy, biofilms were detected of about 20 μm in depth and up to 1 mm in length. These contained copious extrapolymeric matrices, diverse morphotypes of microorganisms and debris. Gram-positive bacteria were recovered from all plant species representing ca. 10% (parsley) up to 65% (broad leaved endive) of the total population. No further differentiation to the species level was performed.

The well-known potential of lactobacilli to form extracellular matrices suggests that these bacteria can be components of the biofilms or even build them up. Lactobacilli commonly share the habitat phyllosphere with species of the genera *Leuconostoc*, *Pediococcus* and *Weissella*. Species frequently recovered from the leaves include *L. plantarum*, *L. paracasei*, *L. fermentum*, *L. brevis* and *L. buchneri* (Daeschel et al., 1987; Müller and Lier, 1994). As components of the rhizosphere community, *L. plantarum*, *L. brevis* and *L. fermentum* were reported to occur most frequently (Kvasnikov et al., 1983).

Also reported were small numbers of *L. brevis* and occasionally *L. casei*, *L. viridescens*, *L. cellobiosus* and *L. salivarius* on a wide variety of plants in a subtropical area (Mundt and Hammer, 1968). Little is known about the interdependence of plants and lactobacilli. As discussed by Daeschel et al. (1987), certain LAB protect plants by producing antagonistic compounds (Visser et al., 1986) that contribute to an inhibition of the plant pathogens *Xanthomonas campestris*, *Erwinia carotovora* and *Pseudomonas syringae*. Furthermore, acid is formed which lowers the pH at injured parts and acts additionally in preventing growth of opportunistic phytopathogens. On the other hand, phytoncides (antimicrobials from plants) probably do not exert strong effects on LAB, as is indicated by their excellent growth during fermentation of vegetables. A well-known exception is oleuropein and its breakdown products, which inhibit various lactobacilli, pediococci and leuconostocs (Etchells et al., 1975). The compound is degraded especially by strains of *L. plantarum* (Ciafardini et al., 1994). Part of our information about the occurrence of lactobacilli on plants is derived from microbiological studies of the fermentation process. Thus, the microbial population upon initiation of the process is known for cabbage (Buckenhüskes et al., 1986), silage raw materials (Langston and Bouma, 1960a; Langston and Bouma, 1960b; Ruser, 1989), carrots and beets (Andersson, 1984), olives (Lavermicocca et al., 1998) and fruits such as grapes (Weiller and Radler, 1970; Suárez et al., 1994) and pears (Heinzl and Hammes, 1986). Although the numbers detected at this stage do not reflect those of the living plants, they indicate what type of organisms may potentially perform the fermentation process or might become agents of food spoilage.

Soil, Water, Sewage and Manure

The presence of lactobacilli in soil and water depends on the content of fermentable substrates. Thus, they are more frequent in soils (Kvasnikov et al., 1983) in which plants grow and constitute a part of the bacterial plant rhizosphere or are washed off from the phyllosphere. They are further involved in the breakdown of decaying matter. Correspondingly, lactobacilli are not found in fresh or marine waters but occur in sewage. From aquatic sources, a multitude of heterofermentative and homofermentative lactobacilli have been isolated (Weiss et al., 1981). In sewage they were present at numbers of 10^4–10^5 cfu/ml. Heterofermenters made up 25% of the total LAB and comprised strains of *L. fermentum*, *L. reuteri*, *L. brevis*, and to a minor extent, of *Weissella confusa* and *Leuconostoc* species. The major part of the homofermenters consisted of *L. plantarum* and *L. ruminis*, and two new species were recovered from sewage, *L. sharpeae* and *L. agilis*. Also found were *L. casei*, *L. acidophilus*, *L. farciminis*, *L. curvatus*, *L. sakei*, *L. lactis*, *L. salivarius* and *L. corniformis*. The majority of these species has also been isolated from human feces (see above) and may therefore originate from that habitat. The same holds true for *L. curvatus* and *L. corniformis*, which were first isolated from manure (Abo-Elnaga and Kandler, 1965). Similarly, *L. vaccinostercus* has been exclusively isolated from cow dung (Okada et al., 1979).

Food Fermentations

RAW MATERIALS OF PLANT ORIGIN After harvest, plant materials undergo lactic acid fermentation when the content of sugar or starch is high, neutral or weakly acid conditions prevail, and access of oxygen is prevented. Lactic acid bacteria occur furthermore in alcoholic fermentations where they are associated with yeasts.

VEGETABLES AND FRUITS The lactic acid fermentation of vegetables and fruits are traditional processes to protect plant raw materials from spoilage. Further, advantageous effects of the fermentation process include the development of characteristic sensory properties, removal of toxins as well as antinutritional components and improved digestibility (Kandler, 1981). Products and microorganisms involved have been reviewed recently (Nout and Rombouts, 2000; Buckenhüskes, 2001). Most information on lactobacilli relating to these products is available for olives, sauerkraut, pickles, as these are the products of main economic importance in the western world. In addition, a vast multitude of products from various raw materials are fermented which however have only regional importance, for example, kimchi in Korea. The processes are commonly a spontaneous fermentation performed by a fortuitous microbial association. The microbial population of fresh raw materials is dominated by aerobic bacteria and

yeasts, whereas LAB represent only a minor component (see above). During fermentation, under the influence of osmotically active salt, anaerobiosis, death of cells, increasing availability of nutrients released from the plant cells, and drop in pH and redox potential, LAB gain dominance and undergo characteristic qualitative successions. With sauerkraut and pickles as examples, the role of lactobacilli in vegetable fermentation can be described.

For sauerkraut it is well established (Brunkow et al., 1925; Stamer, 1975; Pederson, 1979; Kandler, 1983a; Buckenhüskes et al., 1986; Kandler et al., 1986b; Daeschel et al., 1987) that the succession proceeds via the growth of *Leuconostoc mesenteroides*, representing up to 89.5% of the total LAB after 5 days of fermentation at 19°C. *Lactobacillus sakei*, *L. curvatus*, *L. plantarum* and *L. brevis* may be found at low numbers (W. Schneider and W. P. Hammes, unpublished results). The initial phase is followed by the growth of "betabacteria," mainly *Lactobacillus brevis*, and in rare cases *L. buchneri*. Homofermentative bacteria become dominant thereafter for a period whose length depends on the temperature. They consist mainly of *L. plantarum* (old synonym: *L. cucumeris*; Brunkow et al., 1925; Pederson, 1969), *L. curvatus* and *L. sakei*. Other lactobacilli of minor importance resemble *L. paracasei* (formerly *L. casei* subsp. *pseudoplantarum*). Lactococci, enterococci, and pediococci may also be found, but their numbers are commonly low, usually not exceeding 10% (Kandler et al., 1986b) of the total LAB.

Similar fermentation events take place in the Korean kimchi, which is produced from a mixture of Chinese cabbage, radish, cucumber, onion, pepper, garlic etc. and usually with more salt (up to 5%) than used with sauerkraut (1.5–2.5%). Leuconostocs are again the main organism in the early fermentation phase, and *Leuconostoc kimchii* (Kim et al., 2000a), and *Leuconostoc gelidum* (Kim et al., 2000b) were isolated from these products and described as new species. In addition, *Lactobacillus kimchii* was isolated by Yoon et al. (2000) from kimchi. This species is very closely related to and may be even identical with *L. paralimentarius* (see Fig. 6 and Table 6).

For the fermentation of olives and cucumbers, whole fruits or vegetables in brine are used. Therefore, the nutrients are not as readily available as in sauerkraut and silage, where they are released by shredding and chopping, respectively. As shown by Daeschel et al. (1987), LAB can grow not only in the brine, but after brining also within the plant tissue, where they likely enter via the stomata of the epidermis. The

succession of LAB in cucumber fermentation resembles that of sauerkraut (Pederson, 1979). Etchells et al. (1975) observed growth of LAB in the following order of increasing prevalence: *Leuconostoc mesenteroides*, *Enterococcus faecalis*, *Pediococcus cerevisiae*, *Lactobacillus brevis* and *L. plantarum*. As pointed out by Daeschel et al. (1987), *Leuconostoc mesenteroides* is undesirable from a product viewpoint since the CO_2 produced contributes to gas spoilage. The organism is less salt and acid tolerant and prevails only shortly in the initial fermentation phase. Kandler (1983a) has observed that *L. curvatus* and *L. sakei* are also important species in cucumber fermentation. A predominant role of *L. plantarum* has also been observed in the various types of olive fermentation. Recent reviews of these processes and the microorganisms involved have been presented by Harris (1998) and Garrido-Fernandez et al. (1995). The strains occurring on the olive leaves or in the fermentation brines are rather resistant to oleuropein, which is the bitter principle of olives. Some strains of *L. plantarum* can even utilize the compound by splitting off the aglycon and fermenting the glucose moiety (Lavermicocca et al., 1998). In addition to *L. plantarum*, *Leuconostoc mesenteroides*, enterococci and yeasts usually occur in the indigenous processes.

The fermentation of juices of fruits and vegetables is a new method to obtain beverages of appealing flavor. The juices are pasteurized and fermented with the aid of starter cultures (see below).

CEREAL PRODUCTS The production of bread requires leavening of the dough. Before yeasts were available for bakeries, sourdough was the only biological leavening agent (Spicher, 1983). Today, breads from wheat may be leavened with yeast exclusively but sourdough or liquid preferment containing lactobacilli (*L. fermentum*, *L. plantarum* and others; Miller and Johnson, 1958) are also in use. In addition to leavening, the application of sourdough improves the flavor and shelf life of bread. For Italian sweet baked goods of the panettone type, the leavening is the major desired effect of the sourdough. Breads made from rye flour or mixtures of wheat and more than 20% of rye require acidification of the dough, which is traditionally performed by the addition of sourdough. Chemical acidification in combination with yeast as leavening agent is alternatively practiced. Traditionally, sourdough contains yeasts and lactobacilli that have formed a mixture of adapted organisms in the course of their continuous propagation. The role of LAB in sourdough was first recognized by Holliger (1902). The description of the organisms

reflected the state of taxonomy and isolation techniques of that time. The importance of heterofermentative lactobacilli was suggested by Henneberg (1909). Investigation of Danish sourdoughs led Knudsen (1924) to the conclusion that "Betabacterium γ" is a highly adapted organism which is the true sourdough bacterium. From his description, it can be deduced that Betabacterium γ is identical with *L. sanfrancisco*, first described in San Francisco sourdough bread by Kline and Sugihara (1971). More recent studies of the microorganisms in sourdough revealed the presence of yeasts and of homofermentative and heterofermentative lactobacilli: *L. delbrueckii*, *L. acidophilus*, *L. plantarum*, *L. casei*, *L. farciminis*, *L. homohiochii*, *L. brevis*, *L. buchneri*, *L. fermentum*, *L. hilgardii*, *L. sanfrancisco* and *L. viridescens* (Spicher and Loenner, 1985; Spicher, 1987). The microbiology of sourdough and its application has been reviewed by Spicher (1983) and Sugihara (1985).

For the production of soda crackers in the United States, a *Lactobacillus*-yeast fermentation of the dough is used. Sugihara (1978) detected *L. plantarum* as the dominant species in the dough and *L. delbrueckii* in significant numbers. From these species, a pure starter preparation has been developed for improved control of the fermentation process.

Similar fermentation processes take place in numerous doughs or batters of indigenous fermented foods made from cereals or other starch containing raw materials that are not baked but simply boiled or steamed or even consumed without any heating. *Leuconostoc* and *Enterococcus* are usually important fermentation agents in addition to lactobacilli. These products are discussed by Steinkraus (1983), Wood and Hodge (1985b), and Holzapfel (1989).

SILAGE As for the fermentation of foods, the production of silage has a tradition dating back to ancient times. The main agents of the fermentation are lactobacilli, which are therefore of considerable economic importance. Silage is made from various raw materials, of which grass, hay, and maize play the major role. Depending on the quality of the raw material, the dry matter content, and the technology of ensilation, populations of LAB develop which determine the final quality of silage. Since clostridia can multiply at pH values above 4.2, it is important to decrease the pH below that value (Groß and Riebe, 1974). This limit depends, however, on water activity and therefore may be higher when the dry matter content is high. With poorly acidified silages, *Listeria monocytogenes* may also cause hygienic problems (Woolford, 1984). Only after streptococci and leuconostocs have multi-

plied, do lactobacilli and pediococci dominate the silage microflora (Langston et al., 1962). Species chiefly isolated have been *Lactobacillus plantarum*, *L. casei*, *L. brevis*, *L. buchneri*, unclassified "streptobacteria," *L. coryniformis*, *L. curvatus*, *L. casei*, *L. fermentum*, *L. acidophilus* and *L. salivarius* (Keddie, 1959; Langston and Bouma, 1960b; Langston and Bouma, 1960a; Abo-Elnaga and Kandler, 1965; Azeezullah et al., 1973; Grazia and Suzzi, 1984; Dellaglio and Torriani, 1986). Thorough investigation of the LAB involved in fermentation of grass and red clover, ensiled as fresh or prewilted material, was performed by Beck et al. (1987). Totally, 612 isolates were characterized and their role in the course of fermentation was evaluated. Leuconostocs and "*Lactobacillus coprophilus*" were the dominant bacteria at the initial phase, after which other species gained importance. Pediococci, *L. plantarum* and *L. graminis*, together with unclassified heterofermentative lactobacilli, persisted for up to 90 days, whereas the organisms of the initial phase died off. There is increasing use of starter cultures for production of silage (Pahlow and Honig, 1986; Seale, 1986).

RAW MATERIALS OF ANIMAL ORIGIN In modern technologies, the fermentation of raw materials of animal origin is characterized by a widely accepted application of starter cultures. Therefore, these aspects are included under the section Technical Applications.

FERMENTED MILKS A multitude of different types of fermented milks is produced worldwide (reviewed by Oberman, 1985). Products containing lactobacilli, produced with the aid of starter cultures, and known to the western world are yogurt, kefir, and some dietary products, of which acidophilus milk is most representative. Starter cultures for yogurt production contain *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. These bacteria multiply in the milk during incubation at 40–42°C in a symbiotic association (Pette and Lolkema, 1950; Robinson and Tamime, 1981; Marshall and Law, 1984). The *Lactobacillus* species has stronger proteolytic activity, and the amino acids and dipeptides produced by it stimulate the growth of the *Streptococcus* species (Shankar and Davies, 1978), which in its turn reduces the redox potential and forms formic acid that is stimulatory for the *Lactobacillus* cells (Galesloot et al., 1968). Optimum yogurt flavor is also produced by symbiotic associations of microorganisms (Bottazzi and Vescovo, 1969), with *Lactobacillus* being the primary producer. The major flavor impact is derived from acetaldehyde, but acetone and

diacetyl also contribute to the flavor. Threonine was found to be the precursor for acetaldehyde production by *L. delbrueckii* subsp. *bulgaricus* (Lees and Jago, 1978).

Kefir is a product of combined alcoholic and lactic acid fermentation, containing 0.9–1.1% lactic acid and 0.5–1% alcohol. The fermentation is initiated by the addition of kefir grains or starter cultures derived from them. These grains have been found to contain yeast (mainly *Candida kefir*) and LAB. Lactobacilli are the dominant group in the grains, and in addition, leuconostocs and lactococci were also identified (Kunath, 1983). *Lactobacillus acidophilus*, *L. kefir* (Kandler and Kunath, 1983b) and *L. kefiranofaciens* (Fujisawa et al., 1988) are the most characteristic lactobacilli, the latter organism being responsible for production of kefiran, a polysaccharide derived from the matrix of kefir grains (La Rivière et al., 1967; Toba et al., 1986). The microbial population composition changes from that of the grains to that of the ready-to-drink kefir. In the final product, lactococci (*Lactococcus lactis*) prevail and the ratio of *L. acidophilus*/*L. kefir* changes from 0.89/0.11 to 0.2/0.8 (Kunath, 1983).

Acidophilus milk is consumed mainly for its therapeutic effect (Robinson and Tamime, 1981; Fernandes et al., 1987). The taste of the product is rather astringent. *Lactobacillus acidophilus* is known to produce antagonistic compounds, to exert probiotic effects (see above), and to grow poorly in milk. Special care has to be taken to keep the numbers of lactobacilli at a high level, since viable cells are considered essential for the therapeutic activity. Thus, the shelf life is usually restricted to around one week.

Cheese Two groups of lactobacilli can be differentiated on the basis of the time of their growth during the process of cheese making. These are the thermophilic lactobacilli, added deliberately as components of starter cultures, and the mesophilic lactobacilli, which are derived from the environment and emerge during the ripening process. The thermophilic starter cultures are applied for the production of cheese types that require elevated temperatures during the process of curd preparation. For example, the production of Emmental cheese includes cooking the curd grains at 53–57°C. The thermophilic LAB grow best at about 45°C. They survive this step and multiply during the cooling-down period (Auclair and Accolas, 1983). Additional examples of cheese types produced with thermophilic cultures are Gruyere, Gorgonzola, Mozzarella, Cacciocavallo and Provolone (Bottazzi et al., 1973). Species included in starter cultures are *L. helveticus*, *L. delbrueckii* subsp. *lactis* and

subsp. *bulgaricus*, in combination with *Streptococcus thermophilus*. Mesophilic lactobacilli grow in virtually all types of cheese. For example, in cheddar cheese, numbers of 10^6–10^8 are found after 10–60 days and are maintained for 4–6 months. Species involved include *L. casei*, *L. plantarum*, *L. brevis* and *L. buchneri* (Chapman and Sharpe, 1981). For the production of white-brined cheese, starter cultures were applied successfully containing lactobacilli in combination with lactococci, enterococci. leuconostocs, or *S. thermophilus*. Species employed were *L. casei*, *L. plantarum*, *L. helveticus* and *L. delbrueckii* subsp. *bulgaricus* (for review, see Haddadin, 1986).

Fermented Sausages Fermented sausages are made from raw meat and gain their characteristic texture, color, flavor, and above all their resistance to spoilage during a ripening process in which lactobacilli are the essential fermentation agents. Fungi, yeasts, and micrococci or staphylococci may also contribute to the special character of the product (Liepe, 1983; Lücke, 1985; Hammes, 1986). In the traditional way, sugar is added as a substrate for lactobacilli, which convert it mainly to lactic acid, thereby decreasing the pH to values, which prevent the growth of food spoiling or poisoning organisms. Decrease of water activity and addition of nitrite (or nitrate) support this effect (Meisel et al., 1989). Formerly designated as atypical lactobacilli, *L. sakei* and *L. curvatus* are the dominant lactobacilli, but *L. plantarum*, *L. alimentarius* and *L. farciminis* have been also isolated (Reuter, 1970; Reuter, 1975). Starter cultures containing *L. plantarum*, *L. sakei*, *L. curvatus* or pediococci, frequently combined with micrococci or staphylococci, have been developed and are widely applied (Hammes et al., 1985).

Food Spoilage

Acid Foods Lactobacilli play an important role in the spoilage of processed foods. They may exert their effect during processing or especially in the final product. Food products with pH values below 4.1 (ICMSF, 1980; Buckenhüskes et al., 1988) are commonly not sterilized but only pasteurized since the outgrowth of endospores of Bacillaceae is not of concern. Further factors that may protect low-acid foods and especially juices and juice-containing soft drinks are the presence of essential oils or benzoic acid, absence of specific nutrients or growth factors, and in carbonated drinks, CO_2. In the case of underprocessing or leakages of food preserves, juices, and juice-containing beverages, lactobacilli may grow and cause formation of slime, gas,

off flavors, turbidity, and changes in acidity. Species involved are mainly *Leuconostoc mesenteroides* and less commonly *Lactobacillus confusus*, *L. buchneri*, *L. casei*, and *L. plantarum* (Back, 1981). In citrus juices, *L. brevis* and *L. plantarum* can multiply at a pH of less than 3.5 and at a temperature of 10°C (Murdock and Hatcher, 1975; Juven, 1976).

LACTIC-ACID FERMENTED FOODS Lactobacilli are also agents of spoilage of fermented foods. In products obtained by lactic acid fermentation, the effect is commonly exerted during the fermentation process. As a result of the growth of inappropriate strains, the fermentation product may become unacceptable. Examples were found in the formation of red color in sauerkraut caused at elevated pH by *L. brevis* (Stamer, 1975) and formation of bloaters in cucumber fermentation as a result of gas formation by heterofermenters and homofermenters, which form CO_2 from malate (McFeeters et al., 1984).

In cheese, the citrate-utilizing species *L. casei* and the heterofermentative *L. brevis* may produce excessive CO_2, giving rise to unwanted gas pockets in cheese and blowing of packaged cheeses (Fryer et al., 1970; Keller and Jaarsman, 1975). Undesired small cracks ("Boekelscheuren") are formed in Gouda and Edam cheese by *L. bifermentans*, an organism which produces CO_2 and free H_2 (Pette and van Beynum, 1943). Slime-forming strains of *L. plantarum* can multiply in cheese pickling brines, causing ropiness, and salt-tolerant streptobacteria multiplying in rennet cause serious texture and flavor defects in Dutch cheese (Stadhouders and Veringa, 1967). Orange-pigmented strains (*L. plantarum* subsp. *rudensis* or *L. brevis* subsp. *rudensis*) may multiply in hard cheese (Sharpe, 1962) and in white-brined cheese (Chomakov, 1962). Formation of biogenic amines has been observed in cheese, and lactobacilli have been identified as causative agents. *Lactobacillus buchneri* was isolated from high-level-histamine Swiss cheese (Sumner et al., 1985). From Gouda cheese (Joosten and Northolt, 1987), strains of *L. buchneri* and *L. brevis* were isolated which produced histamine and tyramine, respectively. A potential for formation of tyramine and histamine in dairy-related bacteria has been observed also for *L. casei* (histamine) and lactococci (tyramine; Voigt and Eitenmiller, 1978).

Sensory defects of raw fermented sausages have been attributed to the growth of inappropriate species of lactobacilli during the ripening process (Corretti, 1958). These defects may be traced back to mistakes in technology or formulation and are greatly reduced by the use of starter culture preparations. The sensory aberrations included defects in color (loss of color stability, gray or green core, and green surface spots) and flavor. Corretti (1958) isolated from spoiled sausages, strains of *L. plantarum*, *L. delbrueckii*, *L. brevis*, *L. buchneri*, lactococci and *Leuconostoc mesenteroides*. In sausages, the activities of these bacteria may be the same as observed for LAB involved in meat spoilage (see below).

FERMENTED BEVERAGES In fermented beverages obtained by alcoholic fermentation, lactobacilli may contribute to the quality of the product but may also cause spoilage.

WINE Lactobacilli commonly occur in many types of wine, despite the high level of ethanol, the low pH of 3.2–3.8, and the added SO_2 present. They can exert profound effects on the quality of wine. When they are present in high numbers, they produce a combination of the various typical wine defects, the prevalence of which differs, depending on the strains present (Dittrich, 1977). Their ability to metabolize organic acids such as malic, citric and tartaric acids may affect the final product in either a desirable (see Genera *Leuconostoc*, *Oenococcus* and *Weissella* in this Volume) or an undesirable way. In low-acid wines, this process is detrimental and must be controlled. Although most lactobacilli from wines decompose malate, the source of malolactic bacteria is uncertain (Kunkee, 1967). They have been detected only sporadically and in small numbers on grapes and grape leaves and may well be part of the established microbial population of the winery itself. A variety of malolactic fermenting species have been isolated, including *Lactobacillus plantarum*, *L. casei*, unclassified "streptobacteria," and the heterofermentative species *L. brevis*, *L. buchneri*, *L. hilgardii*, *L. trichodes*, *L. fructivorans*, *L. desidiosus* and *L. mali* (Pilone et al., 1966; Nonomura and Ohara, 1967; Peynaud and Domercq, 1967; Peynaud and Domercq, 1970; Barre, 1969; Weiller and Radler, 1970; Chalfan et al., 1977; Maret and Sozzi, 1977; Maret and Sozzi, 1979a; Maret et al., 1979b). The homofermentative species disappear during alcoholic fermentation in favor of pediococci and heterofermentative LAB. The same species of lactobacilli are found in French, Spanish, German, Australian, Californian, and Japanese wines. From high-temperature (40–43°C) fermenting grape musts, however, Barre (1978) has isolated pentose-fermenting "thermobacteria," some of which closely resemble *L. acidophilus*.

The decomposition of tartaric acid in wine is usually connected with severe spoilage of wine. Only a few strains of homofermentative and het-

erofermentative lactobacilli perform this reaction (Krumperman and Vaughn, 1966). Radler and Yannisses (1972) observed that the tartrate-decomposing systems of *L. plantarum* and *L. brevis* differ in metabolic pathway (CO_2, lactate, and acetate are formed by both organisms, but succinate is an additional product of *L. brevis*) and several other criteria.

Other effects of lactobacilli in wine are due to 1) the production of diacetyl from citric acid, a substance which enhances flavor when present in traces, but causes spoilage if present in excess; 2) spoilage such as bitterness occurring together with excess formation of mannitol from fermentation of fructose; and 3) occasional flocculent growth of *L. trichodes* (Amerine and Kunkee, 1968).

APPLE CIDER The indigenous microbial population of an apple cider factory is composed partly of lactobacilli. As with wine lactobacilli, only strains that are adapted to survive a low pH, low levels of carbon and nitrogen compounds, and the presence of increasing ethanol will form part of the permanent population. Many of these selected strains, particularly the heterofermentative species, can metabolize malic and citric acid, as well as quinic acid, a substance present at relatively high levels in apple cider (Carr, 1959). Lactobacilli may multiply in stored ciders, where they bring about several changes. They may take part in the malolactic fermentation, which will often be beneficial to the flavor of the cider; they may also metabolize citrate and pyruvate, yielding acetate, lactate and acetoin. The metabolizing of fructose and quinic acid to acetate, CO_2 and dihydroxyshikimate is of special interest (Whiting, 1975), since the acetate formed is detrimental to flavor (Sharpe, 1981).

Heterofermentative isolates from cider are usually *Lactobacillus brevis*. They have an optimum pH of 4.0–5.0 and metabolize actively only at these low pH values. Fructose is preferentially utilized, glucose often only weakly (Carr, 1959). Slime-forming strains cause ropiness by production of polysaccharide from glucose, fructose or maltose but not from sucrose (Millis, 1951). Homofermentative species are mainly strains of *L. plantarum* and also *L. yamanashiensis* (*L. mali*; Carr et al., 1977), which does not however utilize quinic acid.

BEER In the brewery, lactobacilli may cause spoilage but are also applied for useful purposes. Heterofermentative strains are prevalent in spoiled beer and preferentially ferment maltose. They grow poorly on glucose unless an arginine supplement is present as an additional energy source (Rainbow, 1975). They ferment a narrow

range of carbohydrates, have complex nutritional requirements, and are able to tolerate pH values of 3.8–4.3 found in this environment. Their tolerance to the hop resin humolene, unusual for Gram-positive organisms, and the rapidity with which such resistance is acquired (Richards and Macrae, 1964) suggests that they have become adapted to the brewery as their natural habitat (Sharpe, 1981).

Back (1981) obtained about 1,000 strains of bacteria from contaminated beer. These could be allotted to 13 Gram-positive species belonging to 5 genera and were proven to be obligately or potentially beer-spoiling organisms. The most frequently occurring species were *L. brevis* (28%), *Pediococcus damnosus* (27%), *L. casei* (11%), *L. lindneri* (9%) and *L. corniformis* (6%). In addition, *L. curvatus*, *L. plantarum*, *L. buchneri* and two atypical groups of heterofermentative lactobacilli, one of which contained the new species *L. brevissimile*, were isolated (Back, 1987). The growth of the lactobacilli causes a silky turbidity accompanied by acidity and off flavors because of diacetyl (Scherrer, 1972), while slime-forming strains cause ropiness (Williamson, 1959). As discussed by Back (1987), *L. lindneri* and *L. brevissimile* may pass through filters because of their small size and contaminate the beer in the final container. The latter organism does not cause severe spoilage and is not easy to culture. These species die off in the bottle rather quickly and therefore escaped detection, until recently.

FRUIT MASHES For the production of fruit brandies, mashes of fruits, such as apples, pears, plums and cherries are fermented and distilled. To ensure a "clean" yeast fermentation of the mashes, sulfuric acid is added to decrease the pH to about 3.0. In this environment, highly acidophilic and alcohol-resistant LAB grow and may lower the yield of alcohol or add undesired flavor-active compounds (Heinzl and Hammes, 1986; Kleynmans et al., 1989). Some strains of *L. plantarum* and *L. suebicus* grow at pH 2.5 in the presence of 12 and 14% ethanol, respectively. *Lactobacillus suebicus* grows during and, above all, after the alcoholic fermentation and was found in pear and apple mashes exclusively. Other lactobacilli present at this fermentation phase and exceeding numbers of 10^6 cfu/ml were *L. plantarum*, *L. brevis* and *L. hilgardii*.

GRAIN MASHES During the manufacture of malt whiskey, lactobacilli may multiply and reach high numbers during the fermentation process itself. In contrast to brewing, the malt is not boiled and lactobacilli are often present in the malted bar-

ley. Thermophilic strains may multiply during fermentation when a rich supply of nutrients is readily available. This rapidly depresses the pH to such an extent that the activity of the debranching enzymes and residual amylases of the yeast are inhibited, fermentation is not completed, and a much lower yield of alcohol results (MacKenzie and Kenny, 1965; Simpson, 1968). In addition, detrimental flavor compounds such as hydrogen sulfide may be produced (Geddes, 1986). Species of lactobacilli isolated from distillery fermentations include *L. fermentum*, *L. brevis*, *L. casei*, *L. delbrueckii* and *L. plantarum* (Bryan-Jones, 1975).

FOODS PRESERVED BY ACETIC ACID The application of vinegar as a food preservative is a traditional method of preventing spoilage. Acetic acid is an effective acidulant because it exists in acid foods largely in the undissociated form (pK 4.75), which can pass the cell membrane (Baird-Parker, 1980). Mayonnaise, dressings, and salads are examples of foods whose microbial stability (and taste) is mainly affected by acetic acid. When access to oxygen is prevented, the spoilage is most commonly caused by yeasts and lactobacilli with an exceptionally high tolerance to acetic acid. Highest resistance was found for *L. acetotolerans* (Entani et al., 1986), which grows in fermenting rice vinegar broth and can tolerate 4–5% acetic acid at pH 3.5. In mayonnaise and salad dressings, *L. fructivorans* can cause spoilage (Charlton et al., 1934; Kurtzman et al., 1971) at pH 3.7–3.8. Salads composed of mayonnaise or dressings and ingredients like potatoes, meat, marinated fish, eggs, vegetables, etc. have been investigated by Baumgart et al. (1983). From 81 samples, 6 species of lactobacilli were isolated (in order of prevalence): *L. plantarum*, *L. buchneri*, *L. brevis*, *L. delbrueckii*, *L. casei* and *L. fructivorans*. These organisms are commonly tolerant to the added benzoic and sorbic acid and form gas and off flavors in the product.

The spoilage of marinated fish (mainly herring) involves tolerance not only to acetic acid but also to salt (Meyer, 1965). Lactic acid bacteria (LAB) can grow, cause sensory defects, and form gas that leads to the swelling of containers. Also, CO_2 may originate from fermented carbohydrates or, where these are missing, from decarboxylation of amino acids (Meyer, 1956a). The biogenic amines formed from the respective amino acids are -aminobutyric acid, cadaverine, tyramine and histamine (reviewed by Blood, 1975). The species involved in spoilage are *L. brevis*, *L. buchneri*, *L. fermentum*, *L. pastorianum*, *L. delbrueckii*, *L. plantarum* and *L. casei* (Meyer, 1956a; Meyer, 1956b; Kreuzer, 1957; Lerche, 1960; Yurtyeri, 1963; Reuter,

1965a; Blood, 1970). *Lactobacillus plantarum* and *L. casei* have been also identified as causatives of ropiness in cooked marinades (Priebe, 1970).

SUGAR PROCESSING Of major interest is the effect of LAB during sugar production (Sharpe, 1981). They may cause losses in yield and, owing to excretion of dextran, interfere with the efficiency of the production process and with crystal formation. With cane sugar, most spoilage is caused by leuconostocs. However, sugar-tolerant, acidophilic strains of lactobacilli (able to multiply in 15% sucrose), consisting mainly of *L. confusus* (Sharpe et al., 1972) and occasionally of *L. plantarum* and *L. casei*, multiply in cane juice, causing souring and deterioration of canes. Most of these strains, including *L. plantarum* and *L. casei*, produce large amounts of dextran from the sucrose (Tilbury, 1975). They come from the cane itself and from contaminated equipment. Similar spoilage occurs with beet sugar production (Tilbury, 1975), where strains isolated include *L. casei*, *L. plantarum*, *L. cellobiosus* and *L. fermentum* (Kvasnikov et al., 1976).

MILK Aseptically drawn raw milk contains no lactobacilli when it leaves the udder, but contamination with these organisms rapidly occurs from the dairy utensils, dust, grass, silage, and other feedstuffs. Milk is an ideal substrate for bacterial growth, but conditions that allow contamination and multiplication favor other organisms, and lactobacilli are usually outgrown. In the United Kingdom, single-herd milks produced under good hygienic conditions contain small numbers of lactobacilli, of >1–50/ml, whereas bulked herd market milks usually contain about 10^3/ml (Sharpe, 1981). Species present include *L. casei*, *L. plantarum*, *L. brevis*, *L. coryniformis*, *L. curvatus* and occasionally *L. buchneri*, *L. lactis* and *L. fermentum* (reviewed by Abo-Elnaga and Kandler [1965] and Sharpe [1962]). Raw ewe's milk contains the same species (Chomakov and Kirov, 1975). Pasteurization of the milk (high temperature, short time [HTST] pasteurization, 71.7°C/15 sec) usually destroys all lactobacilli present, except for heat-resistant *L. paracasei* subsp. *tolerans* (Abo-Elnaga and Kandler, 1965). Such heat-treated milks, when used for processing, rapidly become recontaminated from the creamery environment with the same species of lactobacilli (reviewed by Sharpe, 1962). Thereafter, lactobacilli are so universally present in milk and dairy products that generally only strains having unusual characteristics cause spoilage. In liquid milk, slime-producing strains of *Lactobacillus casei*, *L. brevis*, *L. bulgaricus* and *L. acidophilus* occasionally produce ropi-

ness, and *L. maltaromicus* may produce a malty flavor (Miller et al., 1974).

MEAT AND MEAT PRODUCTS Technologies developed for extending the shelf life of meat and meat products include curing, smoking, and packaging in films of low gas permeability together with applying vacuum or controlled atmosphere and refrigeration. These technologies depress the growth of putrefactive microorganisms but create more or less selective conditions for the growth of lactobacilli, leuconostocs (see Genera *Leuconostoc*, *Oenococcus* and *Weissella* in this Volume), carnobacteria (see below), and *Brochothrix thermosphacta* (Kitchell and Shaw, 1975; Egan, 1983). Their growth results in souring, slime formation, off-odor, and greening (Reuter, 1975). Properties of lactobacilli that lead to their preponderance in meat are: psychrotrophy and tolerance to high CO_2 tensions, to nitrite, to salt, and to low pH values. The numbers of lactobacilli on fresh meat and meat products are usually below 10^3/g, and less than 10/cm^2 of the meat surface can grow under the selective conditions present in vacuum packages (Egan, 1983). During cold storage, these atypical lactobacilli usually become the dominant group, and their identity has been investigated in many studies (Allen and Foster, 1960; Cavett, 1963; Gardner, 1968; Mol et al., 1971; Hitchener et al., 1982; Holzapfel and Gerber, 1986; Schillinger and Lücke, 1986; von Holy and Holzapfel, 1989). It has become evident that meat and meat products are a habitat of LAB hitherto not detected elsewhere. Studies of Reuter (1975) and Shaw and Harding (1984) revealed that lactobacilli dominate the competitive leuconostocs or carnobacteria when acidification and/or reduction of the water activity by drying (or smoking) or addition of salt and nitrite are applied, e.g., in dry raw sausages and bacon.

A thorough investigation of lactobacilli in meat and meat products performed by Reuter (1975) showed that "atypical streptobacteria" are the dominant group in all types of meat products. These have been alloted to *L. curvatus* and *L. sakei* by Kagermeier (1981). In addition, *L. plantarum*, *L. casei*, *L. farciminis*, *L. alimentarius*, *L. brevis* and *L. halotolerans* have been found. The latter has been transferred to the rank of species, as *L. halotolerans*, by Kandler et al. (1983d) since it is not related to *L. viridescens* isolated by Niven and Evans (1957) as a causative agency of greening. The greening defect originates from the formation of H_2O_2, which reacts with myoglobin to form the green pigment choleglobin. Sulfmyoglobin is another green pigment formed by the reaction of myoglobin with H_2S. Lactobacilli that form H_2S have been isolated from meat and contribute in this way to

spoilage of vacuum-packed meat. The genetic information for hydrogen sulfide (H_2S) production is plasmid-encoded (Shay and Egan, 1981; Shay et al., 1988). Formation of H_2S and methyl mercaptan by lactobacilli was also described for strains isolated from spoiled Parma hams (Cantoni et al., 1969). The numbers of lactobacilli in spoiled meats may exceed 10^7 or even 10^8/g in products with added sugar, e.g., raw sausages and vacuum-packed cooked sausages (Reuter, 1975).

The animal source of vacuum-packed meat influences the composition of the microbial spoilage association. Shaw and Harding (1984) observed a preponderance of "non-aciduric streptobacteria" (carnobacteria) in pork and lamb, as opposed to beef where lactobacilli prevail. This observation is in partial accordance with earlier studies reviewed by Kitchell and Shaw (1975), which indicated a preponderance of *Brochothrix thermosphacta* on vacuum-packed lamb. Furthermore, lactobacilli do not belong to the spoilage population of refrigerated chicken meat. This is mainly due to the packaging techniques, which consist usually of wrapping without application of vacuum or controlled atmosphere. In addition, when controlled atmosphere is applied, carnobacteria predominate and lactobacilli are rarely found (see the section The Genus *Carnobacterium* in this Chapter).

Technical Applications

Lactobacilli are frequently used as technical and analytical tools. Of supreme importance is their use as starter cultures, while their application as probiotics (see the section Probiotics in this Chapter) receives growing interest (see below). The large-scale production of lactic acid with the aid of *L. leichmannii* (*L. delbrueckii*) is one of the oldest biotechnical processes for producing chemicals by pure cultures (Buchta, 1983). Finally, still in use is the determination of growth factors (vitamins and amino acids) in complex mixtures using auxotrophic strains of lactobacilli.

STARTER CULTURES The application of starter cultures for the production of fermented foods of plant origin has still not been very successful in practice. According to Fleming et al. (1985), starter cultures in combination with the traditional technology do not influence the fermentation process in a way that superior products or an improvement in the economy of the process can be obtained. A limited application has been found for *L. bavaricus*, which is used to produce "L(+)-sauerkraut." This product is sold in health stores because of its content of L(+)-lactic acid. On the other hand, for the production of fermented vegetable juices, several starter cultures are in use. In this case, it is possible to pasteurize

the raw material and to initiate the fermentation without any competing indigenous flora. Starter organisms for fermentation of vegetable juices have been reviewed by Buckenhüskes and Hammes (1990) and include *L. acidophilus*, *L. bavaricus*, *L. brevis*, *L. casei*, *L. delbrueckii*, *L. helveticus*, *L. plantarum* and *L. salivarius*. For production of juices containing L(+)-lactic acid, *L. bavaricus* and *L. casei* are in use.

Fruit juices have been fermented with the aid of *L. casei* (Wiesenberger et al., 1986). In this process, the flavor was changed owing to degradation of malic acid and formation of L(+)-lactic acid.

The application of LAB in wine production is now receiving commercial application (Krieger, 1989). The main aim is to reduce the content of malate, which is converted to L(+)-lactic acid and CO_2 with a decrease in acidity. In addition, both the stability and the flavor of the wine are improved. Most work has been performed with *Leuconostoc oenos*, but lactobacilli have also been applied. Organisms that are sensitive to the acid- and alcohol-containing environment are added to the must before fermentation (e.g., *L. plantarum*), while more tolerant cultures containing *L. brevis* or *L. casei* have also been added to the wine.

Lactobacilli are also used in breweries. In Germany, a sour wort is fermented at 48–50°C, which is added to the mash at 1–2%, thereby decreasing the pH by 0.2–0.4 pH units. This causes the following desired effects: higher enzyme activity in the mash and better separation of protein during wort boiling, followed by a quick fermentation. The sensory quality of the beer is also improved since it becomes more mellow in taste, lighter in color, and the foam has a higher stability. Finally, the microbial stability is positively affected. Maltose-fermenting strains of *L. delbrueckii* subsp. *delbrueckii* are most suitable. The cultures are propagated in the brewery and contain *L. delbrueckii* subsp. *lactis*, *L. amylovorus*, *L. fermentum* and *L. rhamnosus* and occasionally *L. helveticus* (Back, 1988).

In addition, lactic-acid-fermented types of beer are well known, e.g., Berliner Weisse beer, which is produced with *L. brevis* (K. Wackerbauer, personal communication) and sorghum beer (Haggblade and Holzapfel, 1989), in which the souring process is performed at 48–50°C with a thermophilic *Lactobacillus* strain (probably not *L. delbrueckii*; W. Holzapfel, unpublished observations) as the main fermenting organism. Similarly, the Russian drink called "kwas" is a sour beer made from bread, which obtains acidity from fermentation by homofermentative and heterofermentative lactobacilli.

Modern techniques of breadmaking make extensive use of starter culture preparations, the majority of which are mixed strain cultures. The application of starter cultures in modern baking technology requires the additional use of bakers yeast. The starter cultures commonly contain the organisms present in the sourdough but at varying ratios (Spicher, 1984). Böker and Hammes (1990) observed that in a so-called "pure culture sour," more than 99% of the lactobacilli consisted of two strains of *L. sanfrancisco* and one strain of *L. brevis*. This sourdough culture preparation has been used for over 60 years, and its composition has remained remarkably unchanged in various batches of starter in spite of the use of a nonaseptic production process, which is similar to the traditional method of sourdough propagation. Starter preparations consisting of defined strains are also available. Single strain cultures contain *L. brevis*, *L. sanfrancisco*, *L. delbrueckii* or *L. plantarum*, while multiple strain cultures consist of combinations of *L. plantarum*, *L. sanfrancisco* and *L. fructivorans*, or *L. brevis* and *Saccharomyces cerevisiae* (Sugihara, 1985; Spicher, 1987; Budolfsen-Hansen, 1988).

For silage fermentation, the most frequently applied LAB in starter culture preparations were *L. plantarum* (61%), *Lactococcus lactis* (31%), *L. acidophilus* (23%), *Pediococcus acidilactici* (19%), *L. brevis* (16%), *Enterococcus faecium* (11%), *L. lactis* (6%) and *L. bulgaricus* (5%; Pahlow and Honig, 1986). The cultures should ensure quick acidification to pH values below 4.0 and should not be of the heterofermentative type because of possible losses in nutritional value. Their application should further ensure that spoilage microorganisms do not affect the quality, even after the opening of the silo when oxygen gains access. Present knowledge suggests that the cultures should include fast-growing strains of *L. plantarum* and *P. acidilactici*, one of which possesses cellulase activity (Seale, 1986).

PROBIOTICS Originally referring to a substance(s) from one protozoan stimulating another, and later describing an animal feed supplement(s) beneficial to the host by modification of its GI microbial population, a probiotic is now defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989). Probiotics as dietary and therapeutic adjuncts for humans and animal nutrition are now well established in the market and may provide effective and highly acceptable alternatives to conventional growth promoters and therapeutic agents. Their favorable effect on growth and general health is most probably the result of a combination of factors discussed before in this section. In earlier considerations, Gilliland (1979) has listed four desirable properties of an

organism to be used as dietary adjunct: 1) normal inhabitant of the GI tract; 2) resistance to inhibitory systems in the digestive tract; 3) potential for producing beneficial effects; and 4) retention of viability during storage as dietary preparations.

The present status of probiotics, though still in the early stages of application, has been excellently and comprehensively reviewed by Fuller (1989). Referring to the complexity of the underlying mechanism, he emphasized the vital need for additional (basic) information on antagonism, growth rate in the intestine, and attachment to gut epithelial cells (Fuller, 1989). The species most widely used in probiotic products are mainly intestinal strains of *L. acidophilus*, *L. casei*, *L. helveticus*, *L. lactis*, *L. plantarum*, *L. salivarius* as well as *Enterococcus faecium*, *Enterococcus faecalis*, *Bifidobacterium* species and *Escherichia coli*. Special attention has been given recently to possible antimutagenic effects induced by viable LAB. The clastogenic effects induced by a strong chemical mutagen have been inhibited to about 80% by lyophilized cultures of *L. acidophilus* and *Bifidobacterium* in experiments with small laboratory animals (H. Renner, personal communication). Beneficial effects have also been claimed for nonintestinal strains of *L. bulgaricus*, *Streptococcus thermophilus* (Fuller, 1989) and a combination of mesophilic lactobacilli, including *L. curvatus* and *L. sakei*. The "Nurmiconcept," related in principle to the probiotic approach, involves the introduction of the heterogenous (more or less undefined) microbial cecum population of adult poultry to newly hatched chicks, thereby rendering them immediately resistant to 10^3 to 10^6 infectious doses of *Salmonella* (Pivnick and Nurmi, 1982). Aspects of probiotics in poultry nutrition have been reviewed by Jernigan et al. (1985).

Isolation

Media for the isolation of lactobacilli must take into account the aciduric or acidophilic nature of these organisms and their complex nutritional requirements. In some cases, species have adapted to extreme environmental conditions and can only grow on media that simulate their natural habitat. This includes in some cases even strictly anaerobic growth conditions. All media must contain adequate growth factors, usually with yeast extract as a source of vitamins, as well as peptone, manganese, acetate, and the stimulatory Tween 80. A low pH, ranging between 4.5 and 6.2 favors growth. In some habitats, particular spoilage situations, lactobacilli may constitute the only organisms present; more often, they occur together with other organisms, which may include other LAB and yeasts.

MEDIA When lactobacilli are the majority population, MRS agar (see below; de Man et al., 1960) can often be used for isolation. This medium is discussed in detail by Sharpe and Fryer (1965) and compared with the somewhat similar all-purpose with Tween (APT) medium (Evans and Niven, 1951), which is commonly used for isolating *Lactobacillus viridescens* as well as other lactobacilli and carnobacteria from meat products. The growth of especially fastidious lactobacilli, mainly obligately heterofermentative species, has been found to be supported best by the modified Homohiochii medium described by Kleynmans et al. (1989). These media are generally used for cultivation of lactobacilli and other LAB, and may be regarded as semiselective.

MRS Agar for Isolating and Propagating Lactobacilli (de Man et al., 1960)

Oxoid peptone	10.00 g
Meat extract	10.00 g
Yeast extract	5.00 g
K_2HPO_4	2.00 g
Diammonium citrate	2.00 g
Glucose	20.00 g
Tween 80	1.00 ml
Na acetate	5.00 g
$MgSO_4 \cdot 7H_2O$	0.58 g
$MnSO_2 \cdot 4H_2O$	0.25 g

For 1 liter of medium, dissolve 15 g of agar in 1 liter of distilled water by steaming, add all the above ingredients, and adjust pH to 6.2–6.4. Sterilize at 121°C for 15 min.

APT Medium for Isolating and Propagating Lactobacilli (Evans and Niven, 1951)

Tryptone	10.00 g
Yeast extract	5.00 g
K_2HPO_4	5.00 g
Na citrate	5.00 g
NaCl	5.00 g
Glucose	10.00 g
Tween 80	1.00 ml
$MgSO_4 \cdot 7H_2O$	0.80 g
$MnCl_2 \cdot 4H_2O$	0.14 g
$FeSO_4 \cdot 7H_2O$	0.04 g

For 1 liter of medium, dissolve 15 g of agar in 1 liter of distilled water by steaming, add all the above ingredients, and adjust pH to 6.7–7.0. Sterilize at 121°C for 15 min.

Modified Homohiochii Medium for Isolating and Propagating Obligately Heterofermentative Lactobacilli (Kleynmans et al., 1989)

Tryptone	10.00 g
Yeast extract	7.00 g
Meat extract	2.00 g
Glucose	5.00 g
Fructose	5.00 g
Maltose	2.00 g
Na gluconate	2.00 g
Diammonium citrate	2.00 g
Na acetate	5.00 g
Tween 80	1.00 ml
$MgSO_4 \cdot 7H_2O$	0.20 g

MnSO4 ·4H2O	0.05 g
FeSO4 ·7H2O	0.01 g
Mevalonic acid lactone	0.03 g
Cysteine hydrochloride	0.50 g

For 1 liter of medium, dissolve 15 g of agar in 960 ml of distilled water by steaming, add the above ingredients, and adjust pH to 5.4. Sterilize at 121°C for 15 min and add 40 ml of ethanol per liter.

When lactobacilli occur only as part of a complex population, selective media are required. The most widely used of these in the past is the acetate (SL) medium of Rogosa et al. (1953). In this and other similar media, the selective action is based on a low pH of 5.4, a high concentration of acetate ions (inhibitory to many other organisms), and the presence of the growth stimulatory substance Tween 80. These media are further described and discussed by Sharpe (1960). Care has to be taken in preparing SL medium, particularly with regard to final pH since, if this is higher than 5.4, streptococci may not be inhibited. Dehydrated preparations can be purchased commercially, but the final pH should be checked.

Selective SL Medium for Isolating Lactobacilli (Rogosa et al., 1953)

Trypticase	10.00 g
Yeast extract	5.00 g
KH_2PO_4	6.00 g
Diammonium citrate	2.00 g
$MgSO_4 \cdot 7H_2O$	0.58 g
$MnSO_4 \cdot 4H_2O$	0.15 g
$FeSO_4 \cdot 7H_2O$	0.03 g
Glucose	20.00 g
Tween 80	1.00 ml
Na acetate $\cdot 3H_2O$	25.00 g
Agar	15.00 g

Dissolve the agar in 500 ml of water by boiling. Dissolve all other ingredients in another 500 ml of water, adjust pH to 5.4 using glacial acetic acid, and mix with the melted agar. Boil for a further 5 min and pour plates or distribute the hot medium in convenient amounts in sterile screw-capped bottles; no further sterilization is given. Avoid repeated melting and cooling.

This SL medium is recommended for isolation of a wide range of lactobacilli. However, the common meat-spoiling species *L. viridescens* and other species adapted to very acidic environments will not grow. Streptococci, carnobacteria, and other organisms are inhibited, but most pediococci and leuconostocs (from dairy and fermented vegetable sources), some enterococci and bifidobacteria (from intestinal sources), and yeasts may grow. As these pediococci and leuconostocs have metabolic characteristics in common with lactobacilli and many cause similar changes in a product, their detection may be useful (Sharpe, 1962). Growth of yeasts may be eliminated by addition of cycloheximide at a concentration of 10 mg/liter.

Oral Cavity, Intestine and Vagina SL medium was designated initially for selective isolation

of lactobacilli from oral and intestinal sources (Rogosa et al., 1951) and has remained the medium of choice. Some bifidobacteria and occasional enterococci may also grow, and colonies may have to be further identified. Dashkevicz and Feighner (1989) suggested a medium for the detection of bile-salt hydrolase active lactobacilli based on SL or MRS medium supplemented with taurocholic or taurochenodeoxycholic acid. Care should be taken to ensure anaerobic growth conditions. Best results are obtained by application of the Hungate technique.

Milk and Dairy Products SL medium is used for isolation of lactobacilli from milk, cheese, and fermented milks. Cheese starter lactococci are completely suppressed when enumerating cheese samples. Leuconostocs and pediococci, often found in milk and cheese, are not inhibited and colonies may have to be further identified. SL may not be optimum for some thermophilic lactobacilli from dairy sources. Therefore, SL supplemented with 0.5% meat extract is recommended. For selective isolation of *L. delbrueckii* subsp. *bulgaricus* from yogurt, M16 agar (Terzaghi and Sandine, 1975) with pH adjusted to 5.6 with 1.0M acetic acid, has been used successfully (Davies et al., 1977). The isolation and cultivation of *L. kefiranofaciens* from kefir requires the use of KPL medium (Toba et al., 1986).

KPL Medium for Isolating *L. kefiranofaciens* (Toba et al., 1986)

Lactic acid whey	930.0 ml
White table wine	70.0 ml
Glucose	10.0 g
Galactose	10.0 g
Tween 80	1.0 ml
Agar	15.0 g

Mix all ingredients except the wine, adjust pH to 5.5, and boil to dissolve the agar. Sterilize at 121°C for 15 min. To prepare the lactic acid whey, adjust 10% skim milk to pH 5.5 using lactic acid, and boil for 30 min. Remove precipitate by filtration. The table wine (preferentially SO_2-free) is filter-sterilized and added to the autoclaved medium.

For broth medium, use deproteinized lactic acid whey prepared as follows: Adjust 10% skim milk to pH 5.5 using lactic acid and boil for 30 min. Remove precipitate by filtration. Adjust filtrate to pH 7.0 with 2N NaOH and boil for 30 min. Remove precipitate by filtration and readjust pH to 5.5 using 2N HCl.

Meat and Meat Products The use of APT rather than MRS for isolation of *L. viridescens* and other lactobacilli from meat is probably traditional rather than necessary, as these organisms also grow profusely on MRS. Kitchell and Shaw (1975), discussing media for isolation from meats, suggest MRS in addition to APT, incorporating 0.1% thallous acetate with the pH

adjusted to 5.5, or SL adjusted to pH 5.8. A sorbic acid medium was recommended by Reuter (1968) for the selective enumeration of lactobacilli from meat and meat products. Enterococci, leuconostocs, and pediococci may be observed as small (pin-point) colonies and thus can be distinguished from the more prolifically growing lactobacilli.

Selective Sorbic Acid Medium for Isolation of
Lactobacilli from Meat and Meat Products (Reuter, 1968)

Trypticase	10.00 g
Meat extract	10.00 g
Yeast extract	5.00 g
Glucose	20.00 g
Tween 80	1.00 ml
Na acetate	5.00 g
Na citrate	3.00 g
$MgSO_4 \cdot 7H_2O$	0.20 g
$MnSO_4 \cdot 4H_2O$	0.05 g
Agar	20.00 g

To prepare 1 liter of medium, add 0.4 g of sorbic acid as a slightly alkaline solution in water to 1 liter of distilled water. After dissolving the ingredients at 100°C, the pH is adjusted to 5.0 at 50°C. The medium is heated to 100°C, filtered through cotton wool, and boiled again for 30 min.

A modification of the sorbic acid medium, using 0.2% potassium sorbate and a higher pH of 5.7, was found to be especially favorable for the recovery of large numbers of lactobacilli from meat products (Holzapfel and Gerber, 1986). This medium is based on MRS. The commercially available product can be used, to which the 0.2% potassium sorbate is added after boiling and adjusting the pH. Autoclaving is optional since boiling for 30 min is sufficient.

Fermented Vegetables and Silage For silage isolates, SL medium is used. For the low-pH, fermented vegetable processes (using cucumber, sauerkraut, olives, etc.), SL medium and the modified Homohiochii medium are suggested.

Fruit Juices For spoilage organisms from orange juice, Juven (1976) recommends APT medium. Murdock et al. (1952), for the same purpose, recommend a medium containing orange serum.

Orange-Serum Medium for Isolation of Spoilage
Organisms from Fruit Juices (Murdock et al., 1952)

Trypticase	10.0 g
Yeast extract	3.0 g
Orange extract	5.0 g
Glucose	4.0 g
K_2HPO_4	3.0 g
Agar	17.0 g

For 1 liter of medium, dissolve ingredients in 1 liter of distilled water, adjust pH to 5.5, and sterilize at 115°C for 15 min, avoiding overheating.

Fermented Beverages Isolations from wine, beer, and fermented grain mashes, where lactobacilli have adapted to extremely specialized environments, require quite different types of media. It may be necessary to include some of the natural substrate to provide any unknown growth factors essential for strains which have become particularly adapted to their environments. Often, tomato juice can replace these specific growth factors. It may be necessary to suppress such aciduric organisms as yeasts, molds and acetobacters (early work cited by Sharpe, 1960) with inhibitory agents.

Wine For isolation of the slow-growing lactobacilli from wines—both those taking part in the malolactic fermentation and spoilage strains—tomato juice and yeast extract are highly stimulatory and should be included in the medium. The pH should not exceed 5.0. An addition of 4–5% ethanol to all media is also highly recommended. Yoshizumi (1975) suggests the following medium:

Tomato Juice Medium for Isolating Lactobacilli from
Wine (Yoshizumi, 1975)

Glucose	10.00 g
Yeast extract	5.00 g
Polypeptone	5.00 g
KH_2PO_4	0.50 g
KCl	0.12 g
$CaCl_2 \cdot 2H_2O$	0.12 g
NaCl	0.12 g
$MgSO_4 \cdot 7H_2O$	0.12 g
$MnSO_4 \cdot 4H_2O$	0.03 g
Bromcresol green	0.03 g
Agar	15.00 g
Canned tomato juice	150.00 ml

Steam the agar in 850 ml of distilled water to dissolve it first. Add the other ingredients and adjust the final pH to 5.0. Sterilize at 121°C for 15 min. A fungistat should be added to inhibit the growth of yeasts. The author suggests adding Eurocidin or Kabicidin (100 mg/liter). If these cannot be obtained, cycloheximide (100 mg/liter) or sorbic acid (1.2 g/liter) has also been used (Chalfan et al., 1977). It is essential to cultivate under anaerobic conditions for isolation from a later stage of fermentation (Yoshizumi, 1975).

For the isolation of lactobacilli from some wines, it is necessary to use a grape-based medium with added yeast extract and a pH of 3.2–4.5, depending on the wine being examined (Castino et al., 1975; Chalfan et al., 1977). The modified Homohiochii medium has been found to support excellent growth of practically all typical lactobacilli associated with wine.

Cider An apple juice-based medium is recommended (Carr and Davies, 1970) consisting of apple juice plus 1% yeast extract, with the specific gravity adjusted to 1.040, and the pH value to 4.8 with NaOH. The addition of 3% agar is necessary to ensure a firm gel.

Beer For isolation of spoilage-causing brewery lactobacilli, many selective media have been used (Hsu and Taparowsky, 1977). Boatwright and Kirsop (1976) described a sucrose medium and confirmed the usefulness of cycloheximide, polymyxin B, and phenyl ethanol in suppressing yeasts and Gram-negative bacteria. This sucrose agar compared favorably with other brewery media and could be used to cultivate a wide range of lactobacilli, but pediococci and leuconostocs also grew.

Sucrose Agar for Brewery Isolates (Boatwright and Kirsop, 1976)

Sucrose	50.0 g
Oxoid peptone	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g
$MnSO_4 \cdot 4H_2O$	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Tween 80	0.1 g
$CaCO_3$	3.0 g
Bromcresol green	20.0 mg
Agar	20.0 g

Steam the agar first to dissolve it in 1 liter of distilled water. Add the remaining ingredients. Final pH is adjusted to 6.2. Sterilize at 121°C for 15 min after distributing in convenient amounts. Microbial inhibitors are added to the molten agar just before pouring plates (i.e., cycloheximide as a filter-sterilized solution to give a final concentration of 10 mg/ml, and 2-phenyl ethanol without dilution or sterilization to give a final concentration of 0.3%).

A double-concentrated MRS medium adjusted with beer to normal concentration before autoclaving can also be used for cultivation of typical beer lactobacilli. Excellent results for recovery of typical beer LAB are obtained with NBB medium (Back, 1980). The preparation is rather complicated and tedious. A commercial ready-to-use product, however, is available.

Grain Mashes MRS agar was found to be unsatisfactory for isolations from grain mash, and a medium based on a mixture of filter-sterilized malt extract and yeast autolysate has been developed (Bryan-Jones, 1975). Further improvement can be achieved by the addition of wheat flour or bran. For the isolation and propagation of lactobacilli from mageu, a traditional sour maize beverage of southern Africa, the following special medium is recommended (Holzapfel, 1989).

Medium for Isolation of Typical Lactobacilli from Mageu (Holzapfel, 1989)

Filtrate of 5% maize meal porridge	1.00 liter
Tween 80	4.00 ml
Whey powder	20.00 g
Wheat flour	10.00 g
$MgSO_4 \cdot 7H_2O$	0.10 g
$MnSO_4 \cdot 4H_2O$	0.05 g
Triammonium citrate	4.00 g
Sucrose	20.00 g
Cysteine hydrochloride	1.00 g
Agar	15.00 g

Adjust pH before sterilization to 6.0. Sterilize at 121°C for 15 min. Incubate at 50°C for 3–5 days under reduced atmosphere. Inclusion of triphenyl tetrazolium chloride facilitates the enumeration of single colonies against the white medium.

Sake For isolation of spoilage lactobacilli from rice wines, the modified Homohiochii medium can be recommended.

Sourdough Highest recovery of LAB from sourdough was obtained with modified Homohiochii medium, which included the following supplements per liter (W. P. Hammes, unpublished observations): 21 g of bakers' yeast presuspended in 100 ml of deionized water and 50 g of wheat bran.

After sterilizing in centrifuge beakers, the medium is centrifuged (15,000 × γ for 15 min) and the supernatant (slightly turbid medium) is aseptically distributed into appropriate containers. For preparing agar plates, mix double-concentrated broth with equal amounts of sterile, hot 3% water agar.

GASEOUS ENVIRONMENT Most lactobacilli grow better either anaerobically or in the presence of increased CO_2 tension, particularly on first isolation. Agar plates should be incubated in an atmosphere of 90% N_2 and 10% CO_2. Surface plating is recommended so that different colonial types can be observed if present, often indicating the presence of more than one species or biotype. For isolation of oxygen-sensitive intestinal lactobacilli, poured, dried plates must be prereduced by overnight incubation, preferably in an anaerobic jar equipped with catalyst and gas-generating kit. Good anaerobic conditions for isolations should generally also be applied where thermophilic lactobacilli can be expected (i.e., raw milk and dairy products, and fermentations at elevated temperatures). When selective media are used, particularly the SL medium, care must be taken not to dry the plates too long or the concentrated acetate at the agar surface may inhibit growth. Isolates from humans, animals, and some dairy products are incubated at 37°C; from other habitats, at 30°C; and from low-temperature sources, at 22°C.

CULTURE MAINTENANCE Once isolated, unless there are special growth requirements, most species of lactobacilli can be cultured in MRS broth (de Man et al., 1960) or maintained for short periods in MRS agar stabs. For anaerobic lactobacilli, 0.05% cysteine hydrochloride or 0.1%

ascorbic acid should be added, the broth steamed just before use, and organisms cultured under 90% N_2 and 10% CO_2. For some strains or species of lactobacilli, particularly heterofermentative ones, a carbohydrate other than glucose, such as maltose, fructose, or a pentose, gives better growth. For fastidious strains, supplements applied for their isolation are required for their culturing, e.g., freshly prepared yeast autolysate and wheat bran for *L. sanfrancisco*. For several other species, the modified Homohiochii medium (Kleynmans et al., 1989) has given satisfactory results.

CONSERVATION OF CULTURES For preservation for 3–6 months, strains can be stored in yeast glucose litmus milk (YGLM) + calcium carbonate (Sharpe and Fryer, 1965; Bryan-Jones, 1975).

To reconstituted skim milk powder or fresh skim milk, add litmus at a final concentration of 0.01%; yeast extract, 0.2%; glucose, 1%; liver extract, 0.25%; and calcium carbonate, 5%. Divide into 10-ml amounts, sterilize at 121°C for 10 min. Before use, tubes should be incubated for 1 week to check sterility.

This medium is not suitable for the acidophilic heterofermentative isolates from wines and cider. Stab cultures in tomato juice agar at pH 5.0 are preferred. Optimum conditions for medium-term conservation (at least one year) are obtained by mixing a well-grown culture with an equal volume of sterile glycerol and storing at –20°C. Loops of inoculum can thus be taken without exposing the cells to the killing effect of the freezing/thawing procedure.

For long-term preservation, lyophilization is an excellent method for maintaining lactobacilli. Using the method described by Phillips et al. (1975), centrifuged packed cells from a vigorously growing broth culture are resuspended in sterile horse serum containing 7.5% glucose and freeze-dried using the standard technique of Lapage et al. (1970). Similar results are obtained by suspending the cells in 10% skim milk. Ampoules are sealed under vacuum and stored at 5–8°C. Both methods generally result in good recovery of cultures even after 20 years (N. Weiss, unpublished observations).

Highest numbers of survivors after conservation are required for the preparation of freeze-dried starter cultures. The general principles rely on the use of cryoprotectants and the prevention of access of oxygen and moisture during preparation and storage. Cryoprotectants have been reviewed by Bousfield and MacKenzie (1976). The composition of the growth medium and the time of harvest of the culture exert profound effects on the competence of cells for freezing. There are no methods that can be generally applied to all strains, so optimum conditions have to be determined for each strain.

Most optimum results are generally obtained by preservation in liquid nitrogen. The cryoprotectants, 5% DMSO or 10% glycerol, are routinely added before freezing. This method is applied especially for maintaining patent strains.

Phylogeny

Analysis of 16S and 23S rRNA sequences (partial or complete) is widely used for studying phylogenetic relationships of bacteria (Ludwig et al., 1998). On the basis of comparative analysis of 16S rRNA sequences, the phylogeny of LAB has extensively been studied in the past (Yang and Woese, 1989; Collins et al, 1991; Schleifer and Ludwig, 1995a; Schleifer and Ludwig, 1995b). Only little correlation between the traditional classification and the phylogentic relatedness of LAB could be observed, and the genus *Lactobacillus* was subdivided into three major phylogenetic groups: the *Lactobacillus delbrueckii* group, the *Lactobacillus casei-Pediococcus* group, and the *Leuconostoc* group (Collins et al., 1991; Schleifer and Ludwig, 1995a). All former lactobacilli of the *Leuconostoc* group are now reclassified as species of the genera *Leuconostoc* or *Weissella*. Thus, the *Lactobacillus* species listed in Table 1 are actually members of the former *L. delbrueckii* and *L. casei-Pediococcus* groups. In an attempt to overcome the poor correlation between traditional classification and phylogeny, Hammes and Vogel (1995) grouped the lactobacilli by combining the groups based on the phylogenetic relatedness with those based on classical group definitions of Kandler and Weiss (1986a). However, the heterogeneity of lactobacilli, especially those of the *L. casei-Pediococcus* group, is still great with regard to fermentation patterns as well as peptidoglycan types and does not permit the definition of homogenous groups. In a review about the phylogeny of the genus *Lactobacillus* and related genera, Schleifer and Ludwig (1995b) provided evidence that the *L. casei-Pediococcus* group is not uniform and should be split into the *L. salivarius* group, *L. reuteri* group, *L. buchneri* group and *L. plantarum* group.

Following these initial studies, the phylogenetic relatedness of lactobacilli was investigated by using the 16S rRNA sequences of all species validly described up to now (C. Hertel, unpublished results). Various data sets differing with respect to the selection of sequences, and sequence positions were used for phylogenetic analyses applying the distance matrix, maximum parsimony, and maximum likelihood methods. As depicted in Fig. 2, the analyses revealed distinct groups and thus confirmed the
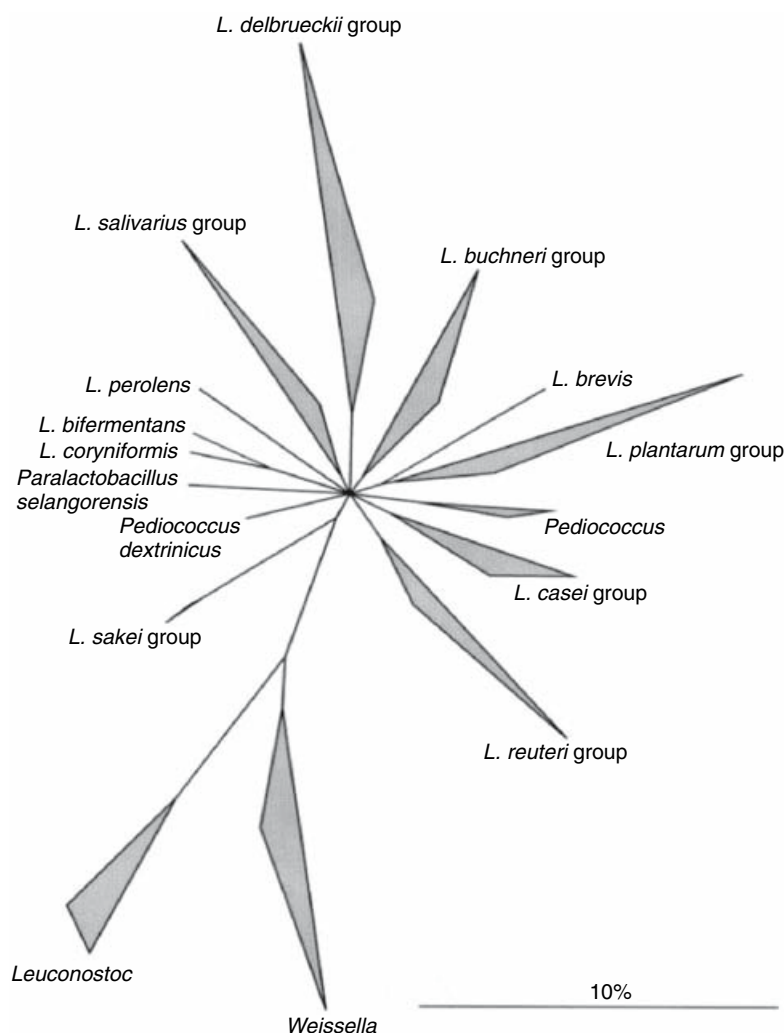
Fig. 2. Phylogenetic tree depicting groups of the family Lactobacillaceae. The consensus tree is based on maximum parsimony analyses of all available, at least 90% complete 16S rRNA sequences of the depicted genera and/or species. The topology was evaluated and corrected according to the results of distance matrix and maximum likelihood analyses with various data sets. Alignment positions that share identical residues in at least 50% of all sequences of the depicted genera were considered. Multifurcations indicate that a common branching order could not be significantly determined or was not supported, when performing different alternative treeing approaches. The bar indicates 10% estimated sequence divergence.

inconsistency of the *L. casei-Pediococcus* group. This study permitted allotment of the lactobacilli to the following groups: *L. buchneri* group (bu), *L. casei* group (ca), *L. delbrueckii* group (de), *L. plantarum* group (pl), *L. reuteri* group (re), *L. sakei* group (sa) and *L. salivarius* group (sl). As the relationship between the groups could not always be resolved unambiguously, the branching is indicated by multifurcations starting from one ancestor. On the other hand, *L. brevis* and *L. perolens* as well as the related species *L. bifermentans* and *L. coryneformis* are uniquely positioned among the lactobacilli. Owing to the extensive research on

lactobacilli, it can be assumed that in the near future related species will be described permitting inclusion of these unique lactobacilli into groups.

In Figs. 3–9, the phylogenetic relatedness within the various *Lactobacillus* groups is depicted. In general, the G+C content of the species within most of the subgroups is rather widespread. This fact may be explained by changes in the codon usages stemming from the degeneracy of the genetic code (Schleifer and Ludwig, 1995b). Furthermore, the peptidoglycan type of the species differed within all groups, except for the *L. delbrueckii* group. In this group, all species

Fig. 3. Maximum likelihood tree reflecting the relationship among the members of the *L. buchneri* group. The tree is based on analyses of all available at least 90% complete 16S rRNA sequences of Lactobacillaceae. Alignment positions that share identical residues in at least 50% of all sequences of Lactobacillaceae were considered. The positioning of *L. parabuchneri* is based on partial sequence data and may be subject to changes. The bars indicate 5% estimated sequence divergence.
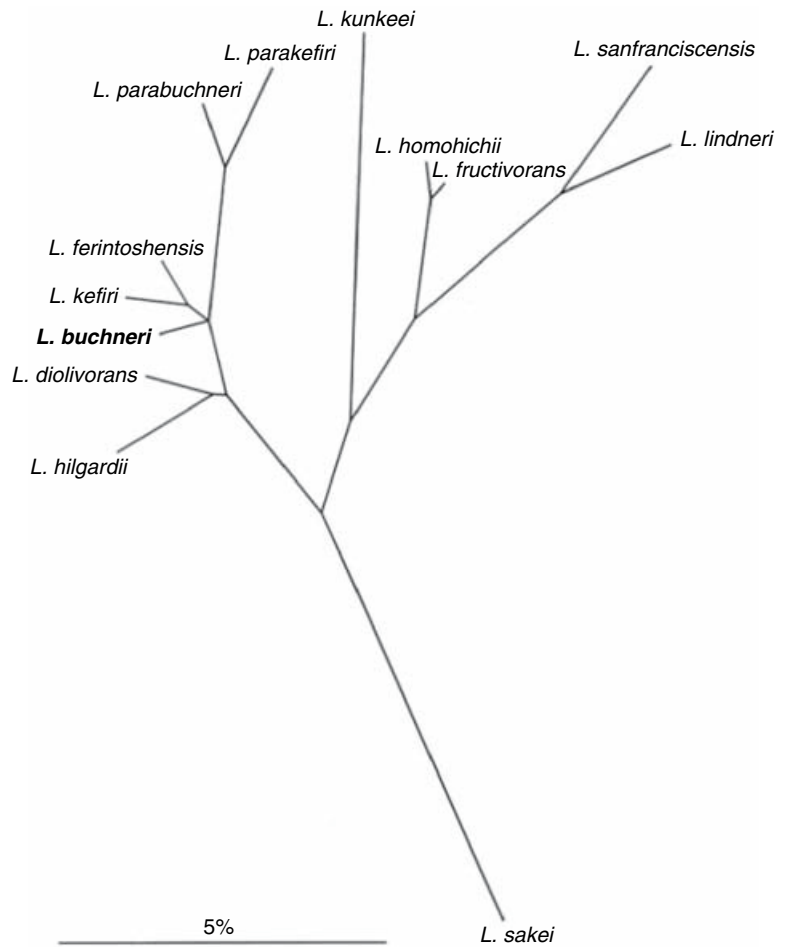
Fig. 4. Maximum likelihood tree reflecting the relationship among the members of the *L. casei* group. Sequences and sequence positions were selected as described for Fig. 3. The bars indicate 5% estimated sequence divergence.
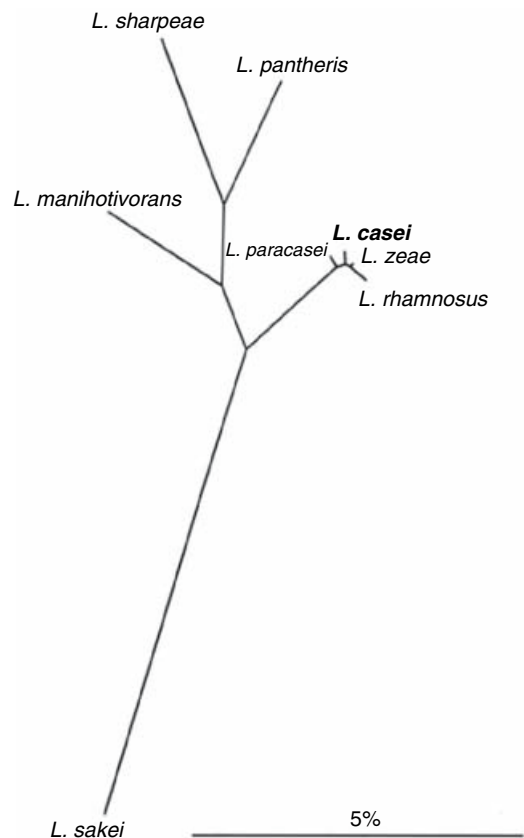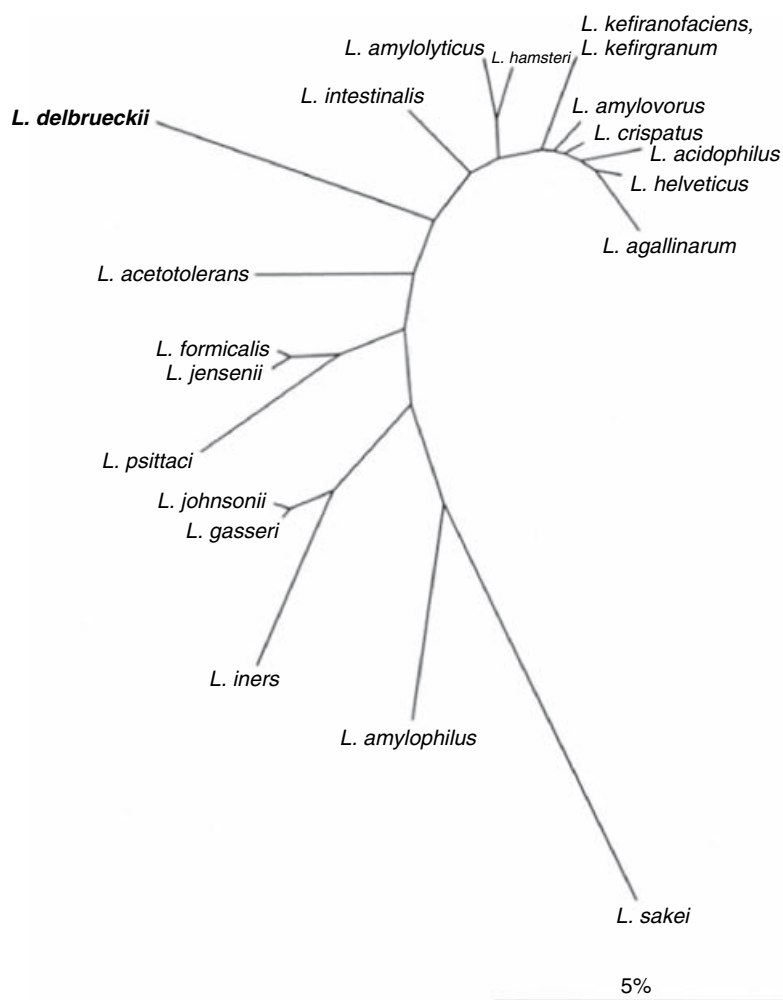
Fig. 5. Maximum likelihood tree reflecting the relationship among the members of the *L. delbrueckii* group. Sequences and sequence positions were selected as described for Fig. 3. The bars indicate 5% estimated sequence divergence.

are of the Lys-D-Asp peptidoglycan type (Schleifer and Kandler, 1972). In all other groups, at least one species exhibits the *meso*-diaminopimelic acid (mDpm)-direct type. Remarkably, a homogenous group with regard to fermentation type is seen only in the *L. buchneri* group, *L. reuteri* group and *L. sakei* group.

The *L. buchneri* group (Fig. 3) contains only obligately heterofermentative lactobacilli, except for *L. homohiochii*, which has been described to be facultatively heterofermentative (Kitahara et al., 1957). Remarkably, the splitting in two evolutionary lines containing as prominent species *L. buchneri* and *L. sanfranciscensis*, respectively, was evident in most of the phylogenetic analyses.

The *L. casei* group (Fig. 4) consists of both obligately homofermentative and facultatively heterofermentative bacteria. The latter are allot-

ted to the highly related species *L. casei*, *L. paracasei*, *L. zeae* and *L. rhamnosus*. Their taxonomic status is currently a controversial subject.

The *L. delbrueckii* group (Fig. 5) is consistent with the former *Lactobacillus delbrueckii* group (Collins et al., 1991; Schleifer and Ludwig, 1995a), which was designated "*L. acidophilus* group" by Schleifer and Ludwig (1995b). It contains mainly obligate homofermenters, and the G+C content of most of the species is <40 mol%. *Lactobacillus delbrueckii* contains three subspecies that cannot be differentiated by rRNA sequence analysis.

The *L. plantarum* group (Fig. 6) consists of 12 *Lactobacillus* species and all three types of carbohydrate fermentation are represented. Remarkably, within this group many species show very high 16S rRNA sequence similarity, namely *L. plantarum*, *L. pentosus* and *L.*

Fig. 6. Maximum likelihood tree reflecting the relation-
ship among the members of the *L. plantarum* group.
Sequences and sequence positions were selected as
described for Fig. 3. The bars indicate 5% estimated
sequence divergence.
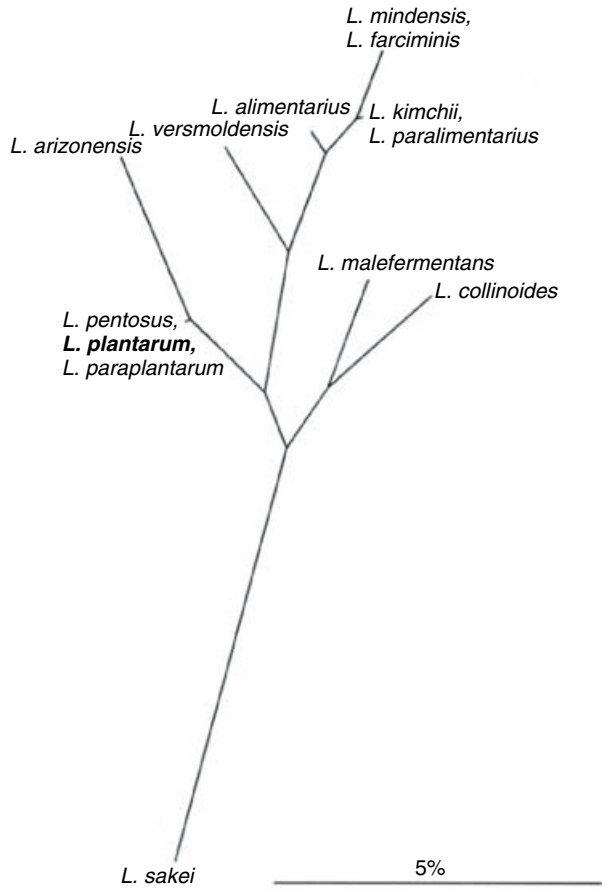
Fig. 7. Maximum likelihood tree reflecting the relationship
among the members of the *L. reuteri* group. Sequences and
sequence positions were selected as described for Fig. 3.
The bars indicate 5% estimated sequence divergence.
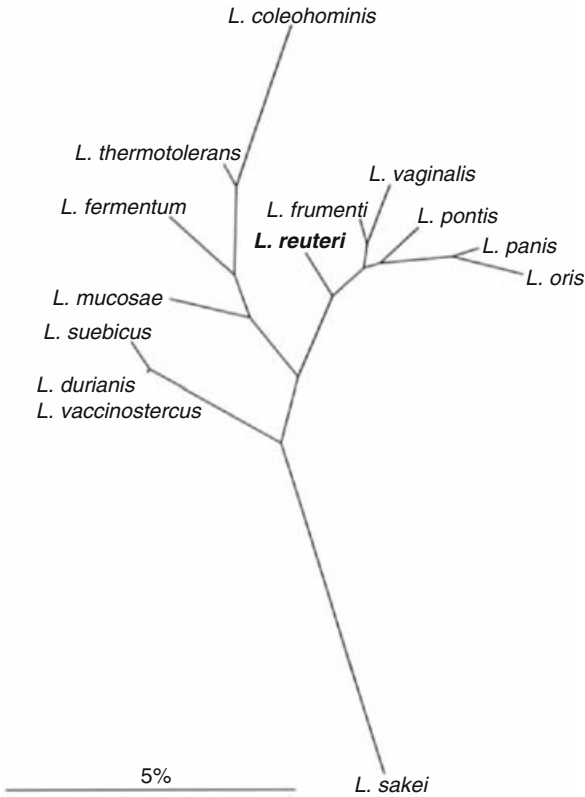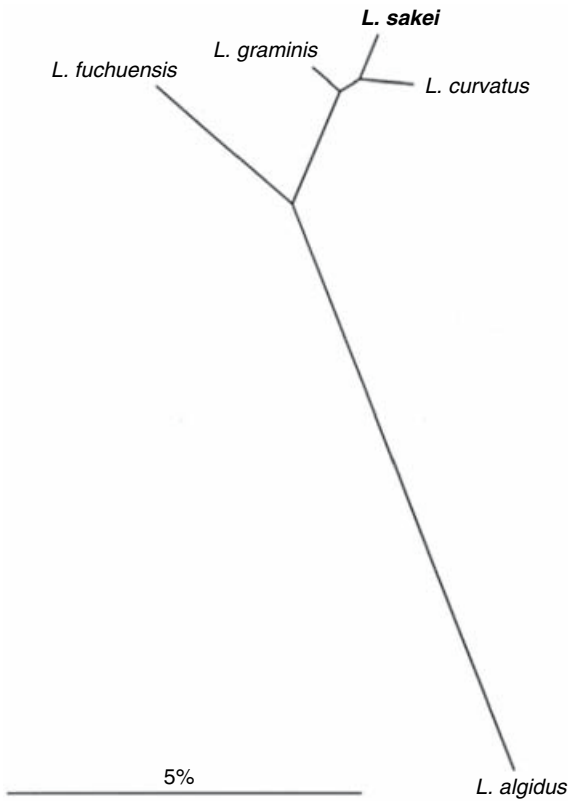
Fig. 8. Maximum likelihood tree reflecting the relationship among the members of the *L. sakei* group. Sequences and sequence positions were selected as described for Fig. 3. The positioning of *L. fuchuensis* is based on partial sequence data and may be subject to changes. The bars indicate 5% estimated sequence divergence.
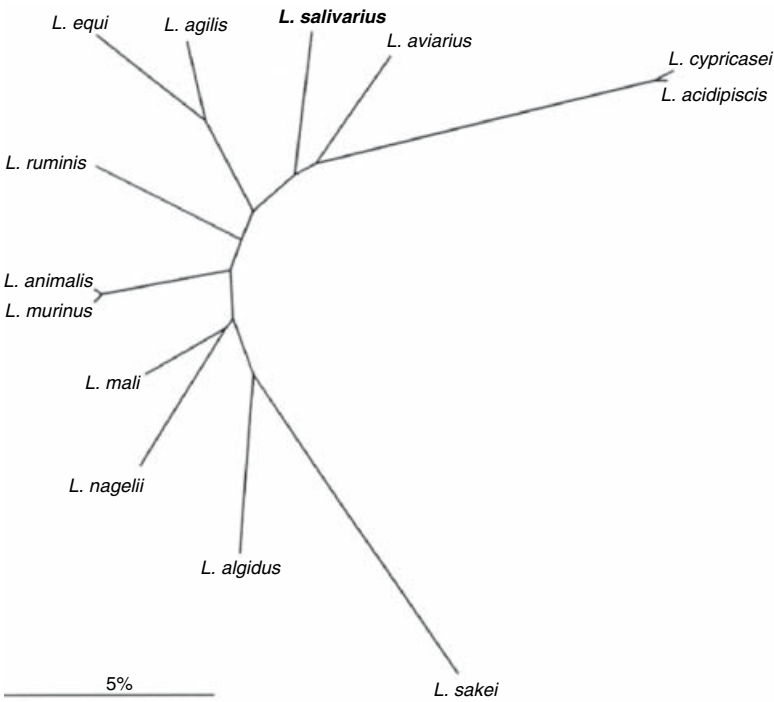
Fig. 9. Maximum likelihood tree reflecting the relationship among the members of the *L. salivarius* group. Sequences and sequence positions were selected as described for Fig. 3. The positioning of *L. acidipiscis* and *L. nagelii* is based on partial sequence data and may be subject to changes. The bars indicate 5% estimated sequence divergence.

paraplantarum (99.7–99.9%), *L. kimchii* and *L. paralimentarius* (99.9%), as well as *L. mindensis* and *L. farciminis* (99.9%), and in addition, their G + C content is rather similar.

The *L. reuteri* group (Fig. 7) contains exclusively obligate heterofermenters, and its members show extraordinary, great differences with regard to their DNA composition (36–54 mol%). The species *L. durianis* and *L. vaccinostercus* exhibit a very high 16S rRNA sequence similarity (99.7%) but were shown to differ significantly in their G+C content (36 and 43 mol%, respectively). Compared to the other highly related species of the genus *Lactobacillus*, this difference in the G+C content is unique.

The *L. sakei* group (Fig. 8) is the smallest subgroup and consists only of facultatively heterofermentative lactobacilli. This subgroup constitutes the deepest branch among the lactobacilli as confirmed by nearly all phylogenetic analyses. Both *L. curvatus* and *L. sakei* contain two subspecies that can only be distinguished by molecular typing methods (Torriani et al., 1996) whose interlaboratory reliability is not given. Thus, the subspecies status is questionable.

The *L. salivarius* group (Fig. 9) contains obligate homofermenters and facultative heterofermenters. Again *L. animalis* and *L. murinus* (99.7%) as well as *L. cypricasei* and *L. acidipiscis*

(99.7%) show 16S rRNA sequence similarities usually found among different species of the genus *Lactobacillus*. *Lactobacillus salivarius* contains two subspecies that cannot be differentiated by rRNA sequence analysis.

Identification

Starting from pure phenotypic methods, genotypic methods are increasingly applied for identification of lactobacilli, and a polyphasic approach is considered to provide the most reliable identification results. Use of this approach allows species that are phenotypically very similar but genotypically quite different to be differentiated, e.g., the former *L. acidophilus* group.

MORPHOLOGY Bacteria sharing habitats with lactobacilli and often growing on the same selective media are *Weissella* spp., leuconostocs, pediococci, bifidobacteria, and occasionally carnobacteria (see below), lactococci, streptococci, enterococci and tetragenococci. These groups can to some extent be morphologically distinguished from lactobacilli. The latter usually occur as rods that may differ in length between the various species (for examples, see Figs. 10A and B, 11A). Some species grow as coccobacilli
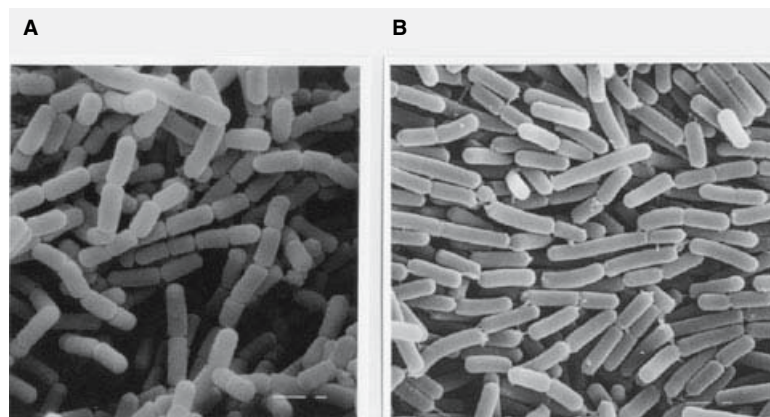
Fig. 10. Electron micrograph of A) L. casei and B) *L. acidophilus* (7000 ×; courtesy of Vittorio Bottazzi).
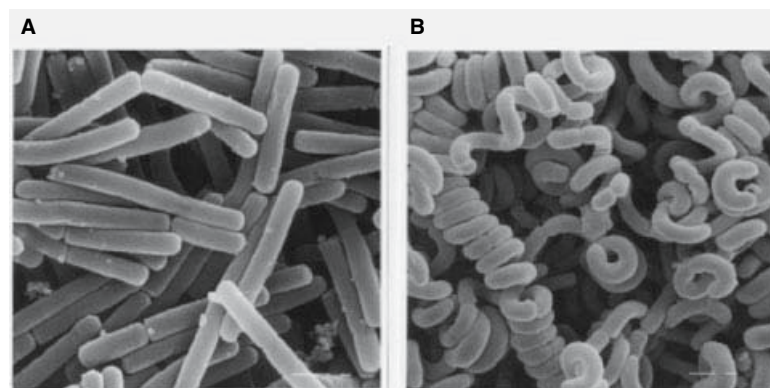
Fig. 11. Electron micrograph of *L. delbrueckii* subsp. bulgaricus. A) cells grown in MRS broth; and B) cells in spiral from MRS colonies (7000 ×; courtesy of Vittorio Bottazzi).

or appear curved or coryneform. Especially some heterofermentative lactobacilli may appear coccoid and can be confused with leuconostocs. Some homofermentative anaerobic lactobacilli from intestinal sources may resemble morphologically certain bifidobacteria. They can be differentiated by their fermentation end products formed from glucose. Whereas the homofermentative lactobacilli produce mainly lactic acid, bifidobacteria are heterofermentative and form acetic acid and lactic acid in a ratio of about 2 : 1. Morphological variations may occur within some *Lactobacillus* species. For example, cells of some freshly isolated strains of *L. sakei* from meat form very short rods and only partially return to bacillary growth after subculturing. Furthermore, as shown by Bottazzi (1988), *L. delbrueckii* subsp. *bulgaricus* can form long spirals (Fig. 11B). Similarly, spiral shapes were also found for *L. curvatus*, which typically forms curved cells.

DETERMINATION OF SPECIAL PHENOTYPIC CHARACTERISTICS Identification of species of lactobacilli requires, in many cases, the determination of numerous physiological and biochemical characteristics. Key properties for differentiation of the species belonging to the different fermentation types are compiled in Tables 5, 6 and 7. Carbohydrate fermentation patterns may be determined either by the conventional test tube method or by commercially available miniaturized rapid systems. Results may not always be identical and sometimes vary from laboratory to laboratory. In addition, some species exhibit a large strain-to-strain variability in these tests, which may partly be explained by the encoding of specific properties on plasmids. In our experience, a reliable identification requires the determination of additional and less variable characteristics. Some tests are described in more detail by Kandler and Weiss (1986a) and are summarized as follows: the determination of the isomers of lactic acid produced from glucose can easily be achieved enzymatically with commercial test kits. The presence or absence of *meso*-diaminopimelic acid (mDpm) in the cell wall can also be checked with a minimum of effort for a large number of strains. The determination of the peptidoglycan type is very helpful for the identification of some obligately heterofermentative species (see Tables 7 and 9).

Reliable results are also obtained by determination of the electrophoretic mobility of lactate dehydrogenases (LDH) in starch gels (Gasser, 1970a) or polyacrylamide gels (Hensel et al., 1977). Relative migration distances of various species have been compiled by Kandler and Weiss (1986a) and Fujisawa et al. (1992). This method is especially useful in the separation of the otherwise phenotypically very similar species of the former *L. acidophilus* group. Recently, the use of electrophoretic patterns of peptidoglycan hydrolases with activity against *Micrococcus luteus* was proposed by Lortal et al. (1997) as a new tool for bacterial species identification. Analysis of the hydrolases of 94 lactobacilli belonging to 10 different species revealed patterns highly similar within a species but specific for each species, even for closely related species, e.g., *L. sakei* and *L. curvatus*. The applicability of the method to colonies was successfully demonstrated.

Comparison of whole-cell protein patterns obtained by highly standardized sodium dodecyl-sulfate polyacrylamide electrophoresis (SDS-PAGE; Pot et al., 1994) has proven to be a reliable tool for the differentiation of species and subspecies especially when they can hardly be differentiated by any other method, e.g., the heterofermentative species *L. brevis*, *L. reuteri* and *L. kefiri* (Dicks and van Vuuren, 1987), the former *L. acidophilus* group (Pot et al., 1993; Klein et al., 1998), the former *L. casei* group (Hertel et al., 1993; Klein et al., 1998), and the subspecies of *L. delbrueckii* (Hertel et al., 1993; Gomez-Zavaglia et al., 1999). In addition, the method was found to be useful for grouping large numbers of strains (Tsakalidou et al., 1994). The use of SDS-PAGE for identification of LAB is however hampered by the fact that it provides only discriminative information at or below the species level, requiring a certain degree of pre-identification (Vandamme et al., 1996). Although comprehensive databases of protein patterns of numerous strains of all species are used, problems in identification of new isolates still exist. For example, Gancheva et al. (1999) used SDS-PAGE of whole-cell proteins to analyze 98 strains belonging to phenotypically highly similar species of the *L. delbrueckii* group (Fig. 5). The majority of the species could be differentiated, but poor discrimination was observed between *L. gasseri* and *L. johnsonii* strains as well as some strains of *L. amylovorus* and *L. gallinarum*. A similar method presented by Gatti et al. (1997) may be useful for identification of *Lactobacillus* species. The authors used SDS-PAGE fingerprinting of cell-wall proteins to characterize thermophilic lactobacilli and observed that different species exhibited varying and typical profiles and, in addition, species-specific proteins were identified for *L. helveticus* and *L. delbrueckii*.

Studies on antigenic determinants have not contributed to an improved identification system for lactobacilli within the last two decades. For the older literature, surveys are presented by Sharpe (1981) and Kandler and Weiss (1986a).

Table 5. Phylogenetic grouping and key characteristics of Group A lactobacilli.[a]

	Species	Peptidoglycan type	G+C content (mol%)	Lactic acid isomer(s)	Growth (°C) 15/45	NH₃ from arginine	Amygdalin	Cellobiose	Galactose	Lactose	Maltose	Mannitol	Mannose	Melibiose	Raffinose	Salicin	Sucrose	Trehalose
3	*L. acidophilus*	Lys-DAsp	34–37	DL	–/+	–	+	+	+	+	+	–	+	d	d	+	+	d
7	*L. amylolyticus*	Lys-DAsp	39	DL	–/+	–	d	–	+	–	+	–	+	d	d	d	+	–
8	*L. amylophilus*	Lys-DAsp	44–46	L	+/–	ND	–	–	+	–	+	–	+	–	–	–	–	–
9	*L. amylovorus*	Lys-DAsp	40–41	DL	–/+	ND	+	+	+	–	+	–	+	–	–	+	+	+
10	*L. animalis*	Lys-DAsp	41–44	L	–/+	–	d	+	ND	d	+	–	ND	+	d	+	ND	d
12a	*L. aviarius* subsp. *aviarius*	Lys-DAsp	39–43	DL	–/ND	ND	d	+	d	d	+	–	+	d	+	d	+	+
12b	*L. aviarius* subsp. *araffinosus*	Lys-DAsp	39–43	L(D)	–/ND	ND	d	d	–	–	+	–	+	–	–	+	+	+
20	*L. crispatus*	Lys-DAsp	35–38	DL	–/+	–	+	+	+	+	+	–	+	–	–	+	+	d
23a	*L. delbrueckii* subsp. *delbrueckii*	Lys-DAsp	49–51	D	–/+	d	–	–	–	–	d	–	–	–	–	–	–	d
23b	*L. delbrueckii* subsp. *bulgaricus*	Lys-DAsp	49–51	D	–/+	–	–	d	d	d	–	–	–	–	–	+	+	–
23c	*L. delbrueckii* subsp. *lactis*	Lys-DAsp	49–51	D	–/+	d	+	d	+	d	+	–	+	+	+	+	+	+
26	*L. equi*	ND	38–40	DL	+/–	ND	–	–	ND	+	+	+	d	+	+	–	+	–
27	*L. farciminis*	Lys-DAsp	34–36	L(D)	+/–	+	+	+	+	–	+	–	+	–	–	+	+	+
34	*L. gallinarum*	Lys-DAsp	36–37	DL	+/+	ND	+	+	+	d	+	–	+	–	–	+	+	d
35	*L. gasseri*	Lys-DAsp	33–35	DL	–/+	–	+	+	+	d	d	–	+	d	d	+	+	d
38	*L. helveticus*	Lys-DAsp	38–40	DL	–/+	–	–	–	+	+	d	–	d	–	–	–	–	–
41	*L. iners*	Lys-DAsp	34.4	L	–/ND	–	ND	ND	ND	–	d	–	ND	–	–	ND	–	d
44	*L. johnsonii*	Lys-DAsp	33–35	DL	+/+	ND	+	+	+	d	+	–	+	d	d	+	+	–
45	*L. kefiranofaciens*	ND	34–35	D(L)	–/–	ND	–	–	+	+	+	–	ND	+	+	+	+	d
46	*L. kefirgranum*	Lys-DAsp	34–39	D(L)	w/–	–	–	d	d	+	–	–	+	–	+	d	d	+
52	*L. mali*	mDpm	32–34	L	+/–	–	+	d	+	–	–	+	+	+	–	+	+	+
53	*L. manihotivorans*	Lys-DAsp	48.4	L	+	+	+	+	+	+	+	–	+	–	–	+	+	+
56	*L. nagelii*	mDpm[b]	ND	DL	+/+	–	+	+	+	d	+	+	+	–	–	+	–	+
59	*L. pantheris*	mDpm[b]	52.7	D	+/–	–	–	+	+	–	+	–	+	–	–	+	+	+
69	*L. psittaci*	ND	ND	ND	ND	–	ND	ND	ND	–	ND	–	ND	–	–	ND	ND	ND
72	*L. ruminis*	mDpm	44–47	L	–/d	–	+	+	+	d	+	+	+	+	+	–	+	–
74a	*L. salivarius* subsp. *salivarius*	Lys-DAsp	34–36	L	–/+	–	–	–	+	+	+	+	–	+	+	–	+	+
74b	*L. salivarius* subsp. *salicinius*	Lys-DAsp	34–36	L	–/+	–	+	+	+	+	+	–	–	–	–	+	–	+
76	*L. sharpeae*	mDpm	53	L	+/–	–	–	–	+	+	+	–	ND	–	–	+	+	–

Symbols and abbreviations: +, 90% or more of strains are positive; –, 90% or more are negative; d, 11–89% of strains are positive; w, weak positive reaction; ND, no data available; (), isomers in parenthesis indicate <15% of total lactic acid; and mDpm, *meso*-diaminopimelic acid.

[a]Obligately homofermentative.

[b]W. P. Hammes, unpublished results.

Table 6. Phylogenetic grouping and key characteristics of Group B lactobacilli.[a]

Species	Peptidoglycan type	G+C content (mol%)	Lactic acid isomer(s)	Growth (°C) 15/45	Carbohydrates fermented												
					Amygdalin	Arabinose	Cellobiose	Esculin	Gluconate	Mannitol	Melezitose	Melibiose	Raffinose	Ribose	Sorbitol	Sucrose	Xylose
1 *L. acetotolerans*	Lys-DAsp	35–36.5	DL	−/−	−	−	d	+	−	d	−	−	−	d	−	−	−
2 *L. acidipiscis*	Lys-DAsp	39–42	L(+)	−/−	d	d	−	−	−	d	−	−	−	+	−	d	−
4 *L. agilis*	mDpm	43–44	L	−/+	+	−	+	+	−	+	+	+	+	+	d	+	−
5 *L. algidus*	mDpm	36.8 ± 3	L+	+/−	d	+	d	+	+	−	+	d	d	+	−	d	−
6 *L. alimentarius*	Lys-DAsp	36–37	L(D)	+/−	ND	d	+	+	ND	−	ND	−	−	d	−	+	−
11 *L. arizonensis*	mDpm[b]	48	DL	+/−	ND	d	+	ND	ND	+	ND	+	+	+[d]	+	+	−
13 *L. bifermentans*	Lys-DAsp	45	DL	+/−	−	−	−	−	−	+	+	+	+	+	+	+	−
16 *L. casei*	Lys-DAsp	45–47	L	+/−	+	−	+	−	−	+	+	−	−	+	+	−	−
19a *L. coryniformis* subsp. *coryniformis*	Lys-DAsp	45	D(L)	+/−	−	−	−	d	+	d	+	d	d	−	d	d	+
19b subsp. *torquens*	Lys-DAsp	45	D	+/−	d	−	−	−	−	−	−	−	−	+	−	+	d
21a *L. curvatus* subsp. *curvatus*	Lys-DAsp	42–44	DL	+/−	d	−	+	+	−	d	−	d	d	+	d	d	−
21b subsp. *melibiosus*	Lys-DAsp	42–44	DL/L(D)	+/−	−	−	d	+	+	−	−	+	d	+	−	+	+
22 *L. cypricasei*	ND	ND	ND	−/−	ND	d	+	+	ND	−	−	−	−	d	−	d	−
30 *L. fornicalis*	ND	37	DL	−/−	+	−	+	+	+	d	+	−	−	+	+	+	−
33 *L. fuchuensis*	mDpm	41–41.7	L(D)	+/ND	+	−	+	+	+	−	−	−	−	+	+	−	−
36 *L. graminis*	Lys-DAsp	41–43	DL	+/−	+	−	+	+	−	d	+	d	d	−	−	+	+
37 *L. hamsteri*	Lys-DAsp	33–35	DL	−/ND	ND	+	d	+	+	d	−	d	+	−	+	+	d
40 *L. homohiochii*	Lys-DAsp	35–38	DL	+/−	−	−	d	ND	−	−	−	−	−	d	−	−	−
42 *L. intestinalis*	Lys-DAsp	33–35	DL	−/+	−	−	d	−	ND	d	−	d	d	d	−	−	−
43 *L. jensenii*	Lys-DAsp	35–37	D	−/+	+	−	+	+	−	d	−	−	−	+[d]	−	d	+
48 *L. kimchii*	Lys-DAsp[b]	35	DL	+/−	+	+	+	+	+	−	+	−	+	+	+	+	+
55 *L. murinus*	Lys-DAsp	43–44	L	−/+	d	−	+	+	−	d	−	d	−	−	−	+	−
61a *L. paracasei* subsp. *paracasei*	Lys-DAsp	45–47	L[c]	+/d	+	−	+	+	w	d	+	−	d	+	d	+	+
61b *L. paracasei* subsp. *tolerans*	Lys-DAsp	45–47	L	+/−	+	−	−	+	−	−	−	−	−	−	−	−	d
63 *L. paralimentarius*	Lys-DAsp[b]	37–38	ND	+/−	+	d	+	+	−	−	−	−	d	−	−	−	−
64 *L. paraplantarum*	mDpm	44–45	DL	+/−	+	d	+	+	+	−	+	+	d	+	d	+	+
65 *L. pentosus*	mDpm	46–47	DL	+/−	+	d	+	ND	+	d	d	+	+	+	−	+	+
66 *L. perolens*	Lys-DAsp	49–53	L	+/−	+	d	d	+	+	−	+	−	−	−	d	+	+
67 *L. plantarum*	mDpm	44–46	DL	+/−	+	d	d	+	+	−	+	+	+	+	+	+	d
71 *L. rhamnosus*	Lys-DAsp	45–47	L	+/+	+	d	+	+	+	+	+	−	−	+	+	+	d
73a *L. sakei* subsp. *sakei*	Lys-DAsp	42–44	DL/L(D)	+/−	−	d	d	+	+	−	−	−	−	+	−	+	+
73b *L. sakei* subsp. *carnosus*	Lys-DAsp	42–44	DL/L(D)	+/−	+	d	d	+	+	+	+	−	−	+	−	+	d
80 *L. zeae*	Lys-DAsp	48–49	L(D)	+/+	+	−	+	+	+	+	+	−	−	+	−	+	−

Symbols and abbreviations: refer to footnote in Table 5.

[a]Facultatively heteroferm entative.

[b]W. P. Hammes, unpublished results.

[c]Strains formerly designated *L. casei* subsp. *pseudoplantarum* produce DL-lactic acid.

[d]According to Carlsson and Gothefors (1975), 60 out of 64 strains ferment ribose.

Table 7. Phylogenetic grouping and key characteristics of Group C lactobacilli.[a]

Species	Peptidoglycan type	G+C content (mol%)	Growth (°C) 15/45	NH₃ from arginine	Arabinose	Cellobiose	Esculin	Galactose	Maltose	Mannose	Melezitose	Melibiose	Raffinose	Ribose	Sucrose	Trehalose	Xylose
14	*L. brevis*	Lys-DAsp	44–47	+/–	+	+	–	d	d	+	–	–	+	d	+	d	–	d
15	*L. buchneri*	Lys-DAsp	44–46	+/–	+	+	–	d	d	+	–	+	+	d	+	d	–	d
17	*L. coleohominis*[b]	mDpm	ND	–/+	–	–	–	–	–	d	–	–	–	–	+	–	–	–
18	*L. collinoides*	Lys-DAsp	46	+/–	+	+	–	+	+	+	–	–	+	–	+	–	–	+
24	*L. diolivorans*	ND	40	+/–	ND	+	–	–	+	+	–	–	+	–	+	–	–	+
25	*L. durianis*	ND	43.3	+/–	–	+	–	d	d	–	–	–	–	–	+	+	–	+
28	*L. ferintoshensis*	mDpm[c]	ND	+/–	+	+	d	+	+	+	+	d	d	d	+	+	+	+
29	*L. fermentum*	Orn-DAsp	52–54	–/+	+	d	d	–	+	+	w	–	+	+	+	+	d	d
31	*L. fructivorans*	Lys-DAsp	38–41	+/–	+?	–	–	–	–	d	–	–	–	–	w	d	–	–
32	*L. frumenti*	Lys-DAsp	43–45	–/+	+	d	+	+	+	+	+	d	+	+	+	+	+	+
39	*L. hilgardii*	Lys-DAsp	39–41	+/–	+	d	–	–	d	+	–	d	–	–	+	d	–	–
47	*L. kefiri*	Lys-DAsp	41–42	+/–	+	d	–	–	–	+	–	–	+	+	+	+	–	+
49	*L. kunkeei*	Lys-DAsp	ND	+/–	–	ND	–	–	–	–	–	–	–	w	–	+	–	–
50	*L. lindneri*	Lys-DAsp	35	+/–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
51	*L. malefermentans*	Lys-DAsp	41–42	+/–	+	–	–	–	–	+	–	–	–	–	+	–	–	–
54	*L. mucosae*	Lys-DAsp	46–49	–/+	+	d	–	+	d	+	–	–	d	d	+	+	–	d
57	*L. oris*	Lys-DAsp	49–51	–/d	–	+	d	d	+	+	d	–	+	+	+	+	d	+
58	*L. panis*	Lys-DAsp	48	–/+	+	+	–	+	+	+	+	–	+	+	+	+	–	+
60	*L. parabuchneri*	Lys-DAsp	44	+/ND	+	+	–	–	+	+	ND	d	d	+	–	+	–	–
62	*L. parakefiri*	Lys-DAsp	41–42	+/+	+	+	–	–	+	+	–	d	d	–	+	–	ND	–
68	*L. pontis*	Orn-DAsp	53–55	+/+	+	–	–	ND	d	+	–	d	d	d	+	+	–	–
70	*L. reuteri*	Lys-DAsp	40–42	–/+	+	+	–	ND	+	+	–	–	+	+	+	+	–	+
75	*L. sanfranciscensis*	Lys-Ala	36–38	+/–	–	–	–	–	d	+	ND	d	–	–	d	d	–	–
77	*L. suebicus*	mDpm	40	+/d	ND	+	d	–	+	+	–	–	d	–	+	d	–	+
78	*L. vaccinostercus*	mDpm	36	–/–	–	+	w	–	w	+	–	–	–	–	+	–	–	+
79	*L. vaginalis*	Orn-DAsp	38–41	–/+	ND	–	–	d	+	+	+	–	+	+	d	+	–	–

Symbols and abbreviations: +?, not determined; and for other symbols and abbreviations, refer to footnote in Table 5.

[a]Obligately heterofermentative.

[b]*L. coleohominis* has been found to produce gas from glucose in our laboratory (W. P. Hammes and C. Hertel, unpublished results).

[c]W. P. Hammes, unpublished results.

Table 8. Key for the presumptive identification of obligately homofermentative and facultatively heterofermentative species of the genus *Lactobacillus*.

1.	*meso*-Diaminopimelic acid present in cell hydrolysates	
1.1.	Ribose fermented	
1.1.1.	Growth at 15°C and 45°C	*L. arizonensis*
1.1.2.	Growth at 45°C, not at 15°C	*L. agilis*
1.1.3.	Growth at 15°C, not at 45°C	
1.1.3.1.	Gluconate fermented	
1.1.3.1.1.	Glycerol fermented	*L. pentosus*
1.1.3.1.2.	Glycerol not fermented	
1.1.3.1.2.1.	α-methyl-D-mannoside fermented	*L. plantarum*
1.1.3.1.2.2.	α-methyl-D-mannoside not fermented	*L. paraplantarum*
1.1.3.2.	Gluconate not fermented	*L. algidus*
1.2.	Ribose not fermented	
1.2.1.	Growth at 15°C and 45°C	
1.2.1.1.	Melibiose fermented	*L. arizonensis*
1.2.1.2.	Melibiose not fermented	*L. nageli*
1.2.2.	Growth at 15°C, not at 45°C	
1.2.2.1.	Lactose fermented	
1.2.2.1.1.	Lactic acid isomer L produced	*L. sharpeae*
1.2.2.1.2.	Lactic acid isomer D produced	*L. pantheris*
1.2.2.2.	Lactose not fermented	*L. mali*
1.2.3.	Growth not at 15°C	*L. ruminis*
2.	*meso*-Diaminopimelic acid not present in cell hydrolysates	
2.1.	Ribose fermented	
2.1.1.	Growth at 15°C and 45°C	
2.1.1.1.	Mannitol fermented	
2.1.1.1.1.	Lactic acid isomers L(D) produced	*L. zeae* (*L. casei*)*
2.1.1.1.2.	Lactic acid isomer L produced	
2.1.1.1.2.1.	Rhamnose fermented	*L. rhamnosus*
2.1.1.1.2.2.	Rhamnose not fermented	*L. paracasei* ssp. *paracasei*
2.1.1.2.	Mannitol not fermented	
2.1.1.2.1.	Sucrose fermented	*L. paralimentarius*
2.1.1.2.2.	Sucrose not fermented	*L. fuchuensis*
2.1.2.	Growth not at 15°C, not at 45°C	
2.1.2.1.	Esculin hydrolysed	
2.1.2.1.1.	Melezitose fermented	*L. fornicalis*
2.1.2.1.2.	Melezitose not fermented	
2.1.2.1.2.1.	Sorbitol fermented	*L. hamsteri*
2.1.2.1.2.2.	Sorbitol not fermented	
2.1.2.1.2.2.1.	Galactose fermented	*L. cypricasei*
2.1.2.1.2.2.2.	Galactose not fermented	*L. acetotolerans*
2.1.2.2.	Esculin not hydrolysed	*L. acidipiscis*
2.1.3.	Growth at 45°C, not at 15°C	
2.1.3.1.	Esculin hydrolysed	
2.1.3.1.1.	Gluconate fermented	*L. hamsteri*
2.1.3.1.2.	Gluconate not fermented	*L. murinus*
2.1.3.2.	Esculin not hydrolysed	*L. intestinalis*
2.1.3.3.	Lactic acid isomer D produced	*L. jensenii*
2.1.4.	Growth at 15°C, not at 45°C	
2.1.4.1.	Mannitol fermented	
2.1.4.1.1.	Melezitose fermented	*L. paracasei* ssp. *paracasei*
2.1.4.1.2.	Melezitose not fermented	*L. bifermentans*
2.1.4.2.	Mannitol not fermented	
2.1.4.2.1.	Xylose fermented	*L. kimchii*
2.1.4.2.2.	Xylose not fermented	
2.1.4.2.2.1.	Melibiose fermented	
2.1.4.2.2.1.1.	NH$_3$ from arginine	*L. sakei*
2.1.4.2.2.1.2.	No NH$_3$ from arginine	*L. curvatus*
2.1.4.2.2.2.	Melibiose not fermented	
2.1.4.2.2.2.1.	Sucrose fermented	*L. alimentarius*
2.1.4.2.2.2.2.	Sucrose not fermented	*L. fuchuensis*
2.2.	Ribose not fermented	
2.2.1.	Growth at 15°C and 45°C	
2.2.1.1.	Melibiose fermented	

Table 8. *Continued*

2.2.1.1.1.	Trehalose fermented	*L. manihotivorans*
2.2.1.1.2.	Trehalose fermented (d)	*L. johnsonii*
2.2.1.2.	Melibiose not fermented	*L. psittaci*
2.2.2.	No growth at 15°C and 45°C	
2.2.2.1.	Galactose fermented	
2.2.2.1.1.	Cellobiose fermented	*L. cypricasei*
2.2.2.1.2.	Cellobiose not fermented	*L. kefiranofaciens*
2.2.2.2.	Galactose not fermented	*L. acetotolerans*
2.2.3.	No growth at 15°C, growth at 45°C	
2.2.3.1.	No growth on acetate containing media	*L. iners*
2.2.3.2.	Lactic acid isomer D produced	
2.2.3.2.1.	Mannose fermented	
2.2.3.2.1.1.	Amygdalin fermented	*L. delbrueckii* ssp. *lactis*
2.2.3.2.1.2.	Amygdalin not fermented	*L. delbrueckii* ssp. *delbrueckii*
2.2.3.2.2.	Mannose not fermented	*L. delbrueckii* ssp. *bulgaricus*
2.2.3.3.	Lactic acid isomer L produced	
2.2.3.3.1.	Mannitol fermented	
2.2.3.3.1.1.	Salicin fermented	*L. salivarius* ssp. *salicinius*
2.2.3.3.1.2.	Salicin not fermented	*L. salivarius* ssp. *salivarius*
2.2.3.3.2.	Mannitol not fermented	*L. animalis*
2.2.3.4.	Lactic acid isomers DL produced	
2.2.3.4.1.	Cellobiose fermented	
2.2.3.4.1.1.	Galactose fermented	
2.2.3.4.1.1.1.	Esculin hydrolysed	
2.2.3.4.1.1.1.1.	Salicin fermented	
2.2.3.4.1.1.1.1.1.	Lactose fermented	*L. gasseri*
2.2.3.4.1.1.1.1.2.	Lactose not fermented	*L. aviarius* ssp. *aviarius*
2.2.3.4.1.1.1.2.	Salicin not fermented	*L. amylovorus*
2.2.3.4.1.1.2	Esculin not hydrolysed	*L. intestinalis*
2.2.3.4.1.2.	Galactose not fermented	
2.2.3.4.1.2.1.	Dextrin fermented	*L. crispatus*
2.2.3.4.1.2.2.	Dextrin not fermented	*L. acidophilus*
2.2.3.4.2.	Cellobiose not fermented	
2.2.3.4.2.1.	Mannitol fermented	*L. equi*
2.2.3.4.2.2.	Mannitol not fermented.	
2.2.3.4.2.2.1.	Lactose fermented	*L. helveticus*
2.2.3.4.2.2.2.	Lactose not fermented	*L. amylolyticus*
2.2.3.5.	Lactic acid isomers L(D) produced	*L. aviarius* ssp. *araffinosus*
2.2.4.	Growth at 15°C, no growth at 45°C	
2.2.4.1.	Lactic acid isomer L produced	
2.2.4.1.1.	Amygdalin fermented	*L. perolens*
2.2.4.1.2.	Amygdalin not fermented	
2.2.4.1.2.1.	Lactose fermented	*L. paracasei* ssp. *tolerans*
2.2.4.1.2.2.	Lactose not fermented	*L. amylophilus*
2.2.4.2.	Lactic acid isomer D produced	*L. coryniformis* ssp. *torquens*
2.2.4.3.	Lactic acid isomers D(L) produced	
2.2.4.3.1.	Mannitol fermented	*L. coryniformis* ssp. *coryniformis*
2.2.4.3.2.	Mannitol not fermented	*L. kefirgranum*
2.2.4.4.	Lactic acid isomers L(D) produced	*L. farciminis*
2.2.4.5.	Lactic acid isomers DL produced	
2.2.4.5.1.	Amygdalin fermented	*L. graminis*
2.2.4.5.2.	Amygdalin not fermented	*L. homohiochii*

[a]*Lactobacillus zeae* and *L. casei* are indistinguishable by phenotypic characteristics.
According to Table 5, 11–89% of strains are positive.

TRADITIONAL PHENOTYPIC SPECIES IDENTIFICATION For the presumptive identification of a *Lactobacillus* strain, dichotomous keys are presented in Tables 8 and 9. The obligately homofermentative and facultatively heterofermentative lactobacilli were combined in one key (Table 8). Therein, the differentiation between the species *L. manihotivorans* and *L. johnsonii* is hampered by the fact that ca. 50% of the strains of *L. johnsonii* ferment trehalose as do

Table 9. Key for the presumptive identification of obligately heterofermentative species of the genus *Lactobacillus*.

1.	*meso*-Diaminopimelic acid present in cell hydrolysates	
1.1.	Arabinose fermented	
1.1.1.	Growth at pH 3.3 and 12% ethanol	*L. suebicus*
1.1.2.	No growth at pH 3.3 and 12% ethanol	*L. vaccinostercus*
1.2.	Arabinose not fermented	*L. coleohominis*
2.	*meso*-Diaminopimelic acid not present in cell hydrolysates	
2.1.	Peptidoglycan of the Lys-DAsp type	
2.1.1.	Ribose fermented	
2.1.1.1.	Growth at 15°C and 45°C	
2.1.1.1.1.	Raffinose fermented	*L. parabuchneri*
2.1.1.1.2.	Raffinose not fermented	*L. parakefiri*
2.1.1.2.	Growth at 15°C, not at 45°C	
2.1.1.2.1.	Mannose fermented	
2.1.1.2.1.1.	Esculin hydrolysed	*L. ferintoshensis*
2.1.1.2.1.2.	Esculin not hydrolysed	*L. parabuchneri*
2.1.1.2.2.	Mannose not fermented	
2.1.1.2.2.1.	Melezitose fermented	
2.1.1.2.2.1.1.	Xylose fermented	
2.1.1.2.2.1.1.1.	Sucrose fermented	*L. buchneri*
2.1.1.2.2.1.1.2.	Sucrose not fermented	*L. collinoides*
2.1.1.2.2.1.2.	Xylose not fermented	*L. parabuchneri*
2.1.1.2.2.2.	Melezitose not fermented	
2.1.1.2.2.2.1.	Melibiose fermented	
2.1.1.2.2.2.1.1.	Galactose fermented	
2.1.1.2.2.2.1.1.1.	Gluconate fermented	*L. diolivorans*
2.1.1.2.2.2.1.1.2.	Gluconate not fermented	*L. brevis*
2.1.1.2.2.2.1.2.	Galactose not fermented	*L. kefiri*
2.1.1.2.2.2.2.	Melibiose not fermented	
2.1.1.2.2.2.2.1.	Xylose fermented	
2.1.1.2.2.2.2.1.1.	NH_3 from arginine	*L. hilgardii*
2.1.1.2.2.2.2.1.2.	No NH_3 from arginine	*L. durianis*
2.1.1.2.2.2.2.2.	Xylose not fermented	
2.1.1.2.2.2.2.2.1.	Fructose fermented	*L. fructivorans*
2.1.1.2.2.2.2.2.2.	Fructose not fermented	*L. malefermentans*
2.1.1.3.	No growth at 15°C, growth at 45°C	
2.1.1.3.1.	NH_3 from arginine	
2.1.1.3.1.1.	Trehalose fermented	*L. frumenti*
2.1.1.3.1.2.	Trehalose not fermented	
2.1.1.3.1.2.1.	Xylose fermented	*L. mucosae*
2.1.1.3.1.2.2.	Xylose not fermented	*L. reuteri*
2.1.1.3.2.	No NH_3 from arginine	
2.1.1.3.2.1.	Ribitol fermented	*L. panis*
2.1.1.3.2.2.	Ribitol not fermented	*L. oris*
2.1.1.4.	No growth at 15°C and 45°C	*L. oris*
2.1.2.	Pentoses not fermented	
2.1.2.1.	Sucrose fermented	*L. kunkeei*
2.1.2.2.	Sucrose not fermented	*L. lindneri*
2.2.	Peptidoglycan of the Orn-DAsp type	
2.2.1.	Growth at 15°C and 45°C	*L. pontis*
2.2.2.	No growth at 15°C, growth at 45°C	
2.2.2.1.	Gluconate fermented	*L. fermentum*
2.2.2.2.	Gluconate not fermented	*L. vaginalis*
2.3.	Peptidoglycan of the Lys-Ala type	*L. sanfranciscensis*

most strains of *L. manihotivorans*. As with any simplified system, a correct species allocation may not be achieved in all cases, and therefore the results should be confirmed by comparison with the characteristics compiled in Tables 5, 6 and 7.

GENOTYPIC IDENTIFICATION Classical genotypic methods include the determination of DNA base composition (mol% G+C) and DNA-DNA similarity studies. The G+C content is known for nearly all *Lactobacillus* species (see Tables 5, 6 and 7) and ranges between 32 and 55 mol%. This

broad range indicates a high genetic heterogeneity within the genus *Lactobacillus*, and commonly, species that differ by more than 10 mol% do not belong to the same genus (Rosselló-Mora and Amann, 2001). Remarkably, this heterogeneity is even found within the various phylogenetic groups of lactobacilli (Fig. 2), e.g., within the *L. reuteri* group containing the species *L. vaccinostercus* (36 mol%) and *L. pontis* (53–55 mol%). The determination of the whole genome DNA-DNA similarity is still an important approach in the current species concept (Rosselló-Mora and Amann, 2001). Such similarity studies have been performed for nearly all species of the genus *Lactobacillus*. An overview of the use of this technique for delineation of *Lactobacillus* species is presented by Vandamme et al. (1996). The DNA-DNA similarity studies were mostly performed to determine new species or to clarify relationships among species but play only a minor role in the rapid identification of unknown isolates.

Owing to the availability of a comprehensive data set of 16S and 23S rRNA sequences, the application of rRNA technology for bacterial identification has become of great importance. For all *Lactobacillus* species, the complete or at least a partial 16S rRNA sequences are available (see Table 1). Comparative analysis of preferably complete or at least sufficiently informative parts of the sequences can therefore be used for the reliable identification of a strain. On the other hand, the 16S rRNA may be too well-conserved to reliably identify closely related species (Fox et al., 1992). Within the genus *Lactobacillus*, several species show very high 16S rRNA sequence similarity, e.g., *L. plantarum*, *L. pentosus* and *L. paraplantarum* (99.7–99.9%), *L. kimchii* and *L. paralimentarius* (99.9%), *L. mindensis* and *L. farciminis* (99.9%), *L. animalis* and *L. murinus* (99.7%), and *L. durianis* and *L. vaccinostercus* (99.7%). Taking these remarks into account, to our experience the comparative analysis of partial 16S rRNA sequences (approximately the first 900 bases) is a fast tool to gain insight into the taxonomic position of an unknown *Lactobacillus* isolate.

Comparative analysis of 16S and 23S rRNA sequences reveals evolutionarily less conserved regions that are diagnostic for species, genus, or groups of phylogenetically related organisms (Schleifer et al., 1993; Amann et al., 1995). Regions containing taxonomically relevant signature positions provide useful target sites for specific probes and primers, which can be used in combination with a variety of hybridization and PCR techniques. The advantages of the use of rRNA targeted probes and their limitations are discussed by Amann and Ludwig (2000). Probes have already been developed for the reli-

able identification of *Lactobacillus* species, and publications describing their successful application can be extracted from the literature. Probes can also be used in the in situ colony hybridization (Ludwig et al., 1995) to identify species in mixed cultures, as it was shown for the detection of lactococci and enterococci species in mixed cultures as well as in fecal samples (Betzl et al. 1990; Hertel et al., 1992; Brockmann et al., 1996). Ehrmann et al. (1994) used reverse dot blot hybridization for simultaneous identification of LAB, and especially lactobacilli in fermented foods. Various species specific 16S or 23S rRNA targeted oligonucleotides were bound to filter membranes and used as capture probes to identify rRNA amplification products generated with universal primers and DNA isolated from cheese, yogurt, sausages, sauerkraut and sourdough. Specific rRNA targeted probes were already used in fluorescent in situ hybridization (FISH) in which the specific rRNA sequences are detected within morphologically intact cells. (To obtain optimal probe permeability for fixed cells, special pretreatment procedures are required for Gram-positive bacteria; Beimfohr et al., 1993.) For example, Lick et al. (2000) constructed two 16S rRNA targeted probes for subspecies differentiation of *L. delbrueckii* by one base-pair difference, which however required the additional identification of the species with a 23S rRNA targeted probe. Matte-Tailliez (2001) constructed 16S rRNA targeted peptide nucleic acid probes for the specific detection of thermophilic lactobacilli cells in milk or starter cultures by FISH. In combination with filtration techniques applied to the samples, the limit of detection ranged between 10^4 and 10^6 cells/ml. Plasmid DNA and (randomly) cloned DNA probes have been developed for strain- and species-specific detection of lactobacilli (Pot et al., 1994). These types of probes may be helpful for the identification of closely related *Lactobacillus* species with nearly identical 16S rRNA sequences. For example, probes targeted against a randomly amplified chromosomal DNA fragment or definite genes (*pyrDEF*) were used for identification of the closely related species *L. pentosus*, *L. plantarum* and *L. paraplantarum* (Bringel et al., 1996; Quere et al., 1997).

The diagnostic regions within 16S and 23S rDNA are also used for the construction of PCR primers. Numerous diagnostic PCR systems for the identification of food-associated and intestinal lactobacilli have been described in the literature. The use of target sites which have already been validated for the application of specific DNA probes facilitate the development of species- or group-specific PCR systems. For example, Bunte et al. (2000) detected *L. paracasei* in fermented sausages and fecal samples by

using a PCR system in which a specific DNA probe was combined with a universal primer. Ward and Timmins (1999) used a combination of specific and universal primers to allot 63 cheese isolates, preliminarily identified as *L. casei*, to the closely related species *L. casei*, *L. paracasei* and *L. rhamnosus*. Other target sequences are helpful to differentiate closely related species or subspecies. For example, Torriani et al. (1999) demonstrated that PCR with primers targeted against the proline iminopeptidase gene (*pepIP*) permit amplification of genomic fragments specific for *L. delbrueckii* ssp. *bulgaricus* or *L. delbrueckii* ssp. *lactis*. In addition, multiplex PCR with several primers was applied for the reliable and rapid identification of lactobacilli. Müller et al. (2000b) developed multiplex PCRs for detection of *L. pontis* and *L. panis* in sourdough fermentations, permitting the combination of species identification with a test of DNA accessibility for amplification. As shown by Yost and Nattress (2000), the combination of various specific primers speeds up the identification. The authors used a multiplex and multistep PCR approach to detect *Carnobacterium* sp., *L. curvatus*, *L. sakei* and *Leuconostoc* sp. in the microbiota of spoiled meat. In addition, Song et al. (2000) developed a two-step multiplex PCR assay for the identification 11 human intestinal *Lactobacillus* species. In the first step, a multiplex PCR was used for grouping of the lactobacilli (4 groups) followed by one multiplex PCR for each group. Thus, up to 4 specific PCRs with 8 different primers were handled in a single reaction.

DNA spacer sequences between the rRNA genes of lactobacilli are highly variable but sufficiently conserved for the derivation of specific PCR primers that may be helpful to identify *Lactobacillus* species (tilsalatimisjävi and Alatossava, 1997; Berthier and Ehrlich, 1998; Chen et al., 2000). Tannock et al. (1999c) used the 16S–23S rRNA gene intergenic spacer region sequence comparison to identify 40 *Lactobacillus* isolates from the gastrointestinal tract (28), silage (2) and probiotic yogurts (10). Confirmation of the identity by sequences of the V2-V3 region of the 16S rRNA gene was obtained in all instances. It was shown that this method can also be used for identification of the closely related species *L. curvatus*, *L. sakei* and *L. graminis* as well as *L. paraplantarum*, *L. plantarum* and *L. pentosus* (Berthier and Ehrlich, 1998). Chen et al. (2000) demonstrated length and sequence polymorphisms in the spacer sequences between the 23S and 5S rRNA genes among the closely related species of the former *L. casei* group indicating the usefulness in identification.

Amplified ribosomal DNA restriction analysis (ARDRA) is based on the restriction length polymorphism of mostly 16S rRNA gene fragments amplified by PCR. The choice of the restriction endonuclease and the length of amplified fragments define the discriminatory power of the method. Isolates obtained from dairy products (yogurt and cheese) were allotted to the species of the thermophilic lactobacilli *L. delbrueckii* and *L. helveticus* by applying ARDRA (Moschetti et al., 1997; Andrighetto et al. 1998; Giraffa et al., 1998; Roy et al., 2001; Bouton et al., 2002). Strains of *L. delbrueckii* were differentiated down to the subspecies level *L. delbrueckii* ssp. *bulgaricus* or *L. delbrueckii* subsp. *lactis/delbrueckii*. On the other hand, some strains of *L. helveticus* could not be identified indicating a potential limitation of the method, especially when applied to such heterogeneous species. The 16S rRNA sequence comparison revealed sequences nearly identical with that of *L. helveticus*, except for one base difference located in the recognition site of the restriction enzyme used for ARDRA, which finally lead to a failure in the identification (Giraffa et al., 2000). Ventura et al. (2000) showed that ARDRA can differentiate between *Lactobacillus* species by using a set of four restriction enzymes and applied the method to identify various *Lactobacillus* isolates from human feces and vagina. Numerical analysis of the ARDRA patterns obtained by using four different restriction enzymes revealed that the method is also an efficient tool for identification of the species of the former *L. casei* group and *L. acidophilus* group (Roy et al., 2001).

Numerous methods were developed for the genotypic typing of bacteria down to the strain level, e.g., ribotyping, random amplified polymorphic DNA (RAPD), pulse field gel electrophoresis, and amplified fragment length polymorphism (AFLP) techniques. With regard to their application to lactobacilli, the reader is referred to the literature. Identification systems were developed which are based on the detection of strain-specific DNA sequences by hybridization or PCR techniques. The unique sequences can be derived, for example, from RAPD fragments (Tilsala-Timisjävi and Alatossava, 1998) or may be obtained by use of subtraction hybridization. The applicability of the latter method to develop strain-specific PCR-based detection systems for food fermenting LAB has been demonstrated (Wassill et al., 1998; Zwirglmaier et al., 2001). Bunte et al. (2000) used this type of PCR system to monitor the fate of strain *L. paracasei* LTH 2579 in fermenting sausages as well as human feces.

DETECTION IN COMPLEX MICROBIOTA Molecular biological methods have been developed for the culture-independent analysis of the diversity

of complex microbial communities. Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA amplicons (Muyzer and Smalla, 1998) has been demonstrated to be a suitable tool for the characterization of microbiota containing lactobacilli. Total bacterial DNA from the habitat of interest is extracted and a variable region of the 16S rDNA is amplified by PCR. The resulting mixture of 16S rDNA fragments is subjected to polyacrylamide gel electrophoresis using a denaturing gradient established with urea and formamide to separate the fragments and generate a genetic fingerprint of the community. The method was applied to monitor the diversity and dynamics of the microbiota during fermentation of maize dough pozol (ben Omar and Ampe, 2000), sour cassava starch (Ampe et al., 2001), sourdough (Meroth et al., 2003), malt whisky (van Beek and Priest, 2002), Italian sausages (Cocolin et al., 2001), water-buffalo Mozzarella cheese (Ercolini et al., 2001), artisanal Sicilian cheese (Randazzo et al., 2002), etc. In these fermenting substrates various *Lactobacillus* species were identified and shown to dominate during the whole fermentation process or in a certain period within the succession of the bacterial population. Use of universal primers permitted the predominant bacteria to be monitored in the fermentation process only, as this method detects 90–99% of the most numerous species in the community. With regard to microbiota in which the lactobacilli represent only a minor part of the population, e.g., the intestinal microbiota, DGGE has to be combined with a *Lactobacillus* group-specific PCR, as demonstrated for example by Walter et al. (2001) and Heilig et al. (2002). For details about the use of molecular biological methods to study the composition and functionality of the intestinal microbiota, the reader is referred to recent reviews, e.g., O'Sullivan (1999), Konstantinov et al. (2002), and McCartney (2002). Recently, the PCR-DGGE was used to investigate the vaginal bacterial microbiota (Burton et al., 2003). The study not only revealed that lactobacilli belong to the dominant group of bacteria but also allowed *L. iners* to be identified as the most common *Lactobacillus* species. This species was hitherto not detected in this microbiota because it could not grow on the major selective media used for isolation of lactobacilli including MRS and Rogosa-Sharp medium (Falsen et al., 1999).

Physiology

The metabolism of lactobacilli appears simple because of their complex nutritional requirements and their fermentative nature. There are, however, certain traits which are characteristic for special groups, species, or even strains that contribute in a unique manner not only to their adaptation to but also their effect on a substrate. Thus, using the various substrates, quite distinct species may be revealed which affect the sensory quality of foods during both the desired fermentation process and the detrimental spoilage process.

Carbohydrate metabolism leads to the production of lactic acid or of lactic acid, CO_2, and acetate and/or ethanol. The homolactic species utilize hexoses by the glycolytic pathway, and the heterofermentative species, by the 6-phosphogluconate pathway (Kandler, 1983a). The latter pathway requires phosphoketolase, an enzyme absent in the obligate homofermenters but present in facultative heterofermentative species. Unusual pathways for carbohydrate metabolism appear to be present in certain groups of lactobacilli. For example, Barre (1978) isolated thermophilic lactobacilli from fermenting grape must that utilized pentoses and performed a homolactic fermentation. The same was observed for isolates related to *L. salivarius* from the intestine of rats (Raibaud et al., 1973) and for an unknown thermophilic species (Fukui et al., 1957). In the latter case, a new fermentative pathway was suggested because sedoheptulose was transported but not fermented by these organisms, which should be the case when the pentoses were degraded via the pentose-phosphate pathway. The fermentable carbohydrates and the products formed both depend on the environment. Thus, glucose represses the activity of ketolases in the facultative heterofermenters, and the pH also exerts effects on the type of fermentable carbohydrates used (Carr, 1987).

The nature of the end products of carbohydrate metabolism is strongly affected by the presence of oxidants (Condon, 1983; Condon, 1987). In the presence of oxygen, lactobacilli transfer electrons to this molecule, leading to the formation of superoxide (O_2^-), H_2O_2, or H_2O. Known enzymatic activities involved are NADH:H_2O_2 oxidase, NADH:H_2O oxidase, pyruvate oxidase, α-glycerophosphate oxidase, and NADH peroxidase. Superoxide dismutase activity has not been found in lactobacilli, although the dismutation of superoxide is catalyzed by internally accumulated manganese (Götz et al., 1980; Archibald and Fridovich, 1981). Hydrogen peroxide can be removed either by NADH peroxidases or by catalases. Catalase activity in lactobacilli depends either on the presence of hematin (Whittenbury, 1964; Wolf and Hammes, 1988) or on a manganese-containing pseudocatalase. True catalase activity has been found in *L. plantarum*, *L. pentosus*, *L. delbrueckii*, *L. sakei*, *L. brevis*, *L. buchneri*, *L. fermentum* (Whittenbury, 1964; Wolf and

Hammes, 1988), and pseudocatalase in *L. plantarum*, *P. pentosaceus*, *E. faecalis*, a *Leuconostoc* species (Johnston and Delwiche, 1962), and *L. mali* (W. P. Hammes, unpublished observations). Another group of oxidants affecting the metabolism of LAB are nitrate and nitrite (Dodds and Collins-Thompson, 1985; Wolf and Hammes, 1988; Wolf et al., 1990). Nitrate reductases have been found in *L. plantarum*, *L. pentosus*, *L. fermentum* and *L. casei* (Costilow and Humphreys, 1955; Langston and Bouma, 1960b; Langston and Bouma, 1960a) and nitrite reductase in *L. lactis*, *L. leichmannii*, *L. buchneri*, *L. plantarum*, *L. acidophilus*, *L. viridescens*, *L. sakei*, *L. farciminis*, *L. pentosus*, *L. brevis* and *L. suebicus* (Fournaud et al., 1964; Collins-Thompson and Lopez, 1981; Wolf et al., 1990). Two types of nitrite reductases are known, those depending on the presence of hematin (*L. plantarum* and *L. pentosus*; Wolf and Hammes, 1988; Wolf et al., 1990) and heme-independent enzymes (*L. delbrueckii* subsp. *lactis*, *L. sakei*, *L. farciminis*, *L. brevis*, *L. buchneri* and *L. suebicus*; Dodds and Collins-Thompson, 1985; Wolf et al., 1990). In the former type of reaction, ammonia is produced, whereas in the latter type, NO and N_2O are the products of nitrite reduction.

The presence of an oxidant leads to the formation of end products of carbohydrate metabolism which are more oxidized than lactic acid, such as CO_2 plus acetate, acetoin, or diacetyl (Condon, 1987). A most instructive example of the effect of oxygen on carbohydrate metabolism was provided by Dirar and Collins (1973), who observed that at low galactose concentration (1–6 mmol/liter), the carbohydrate was degraded by *L. plantarum* to almost exclusively acetic acid (93%) in addition to carbon dioxide. The formation of acetate catalyzed by acetate kinase increases the energy yield in this organism. The same mechanism is active in heterofermenters where acetylphosphate is derived from the reaction catalyzed by phosphoketolase. In the presence of oxygen, the formation of ethanol is reduced in favor of acetate (Kandler, 1983a). In organisms devoid of phosphate acetyltransferase and alcohol dehydrogenase (e.g., *L. brevis*, *L. buchneri*; Condon, 1987), fructose is used as oxidant and is reduced to mannitol. Alternatively, quinic acid is reduced to dihydroshikimic acid by a *L. collinoides* strain isolated from cider, and a similar mechanism was also detected in *L. plantarum* (Whiting and Coggins, 1974). Increased carbohydrate utilization and acetate formation was also observed for *L. brevis* and *L. buchneri* with glycerol and glucose as substrates. Glycerol was reduced to propanediol-1-3 via 3-hydroxypropanal as an intermediate (Schütz and Radler, 1984).

The synthesis of carbohydrate polymers by certain lactobacilli strongly affects the physical properties and visual appearance of a substrate. Thus, slime formation during sugar processing (see above), on surfaces of meat and meat products, in beer (Lawrence, 1988), wine, cider, and soft drinks is a detrimental property of lactobacilli (Williamson, 1959; Dunican and Seeley, 1965; Carr and Davies, 1970). On the other hand, in film jäk (Sharpe, 1979) and yogurt (Davis, 1975), slime formation by LAB is a desired property. The composition of the slimes has been only poorly investigated. The major product is dextran (Sharpe et al., 1972), but heteropolymers are also formed, as, for example, kefiran (La Rivière, 1967).

The metabolism of carbohydrates is the main source of energy and involves substrate-level phosphorylation. In addition to the ATP synthesized during glycolysis, ATP can be derived from acetylphosphate, which is formed in reactions catalyzed by pyruvate oxidase or pyruvate decarboxylase. A further ATP-generating step involves the breakdown of arginine via the arginine deiminase (dihydrolase) pathway, whereby carbamate kinase synthesizes ATP from carbamoylphosphate with the formation of ammonia and CO_2. This mechanism is present in heterofermentative lactobacilli and was studied in *L. buchneri* (Manca de Nadra et al., 1988). A final energy-generating mechanism was proposed by Michels et al. (1979). Their "energy recycling model" postulates that cells can excrete end products together with protons, resulting in generation of a proton motive force that can be used for the production of metabolic energy. The functioning of such a mechanism in *Lactococcus cremoris*, as shown by ten Brink and Konings (1982) and Renault et al. (1988), provided evidence that the performance of the malolactic fermentation serves additionally as a mechanism for building up a proton motive force.

The transport of sugar and its regulation in LAB, in particular in the homofermentative species, have recently attracted interest because of their special advantages as models for studying these metabolic traits (Thompson, 1988a). Unfortunately, the majority of investigations have been performed with lactococci, and only a few lactobacilli were thoroughly studied. Glucose and other saccharides are transported with the aid of permease and are phosphorylated in the cytoplasm. The homofermentative lactobacilli *L. casei* and *L. plantarum* additionally contain a phosphoenol pyruvate (PEP): glucose phosphotransferase system that is missing in heterofermentative species (Romano et al., 1979). The presence of the specific PEP-dependent sugar: phosphotransferase system (PTS) in *L. casei* was detected for ribitol and xylitol (London and Hausman, 1982) as well as lactose (Chassy and Thompson, 1983). In lactose-fermenting lac-

tobacilli, both permease and the PTS appear to be present (Premi et al., 1972), except for *L. casei*, since in five strains of this species, no β-galactosidase but only β-D-phosphogalactosidase could be detected. A plasmid-encoded β-galactosidase was, however, detected in *Lactobacillus casei* ATCC 393 (Chassy, 1987a).

The utilization of proteins and peptides by lactobacilli again is better known for lactococci (*Streptococcus lactis*) than for lactobacilli. The main interest in these metabolic characteristics is derived from the role of LAB in milk and dairy products. Since milk is low in its content of amino acids and peptides, these bacteria have to hydrolyze milk proteins and to transport the resulting peptides. In addition, proteolysis is important in cheese ripening as it affects the texture and flavor in a desired (but sometimes faulty) way (reviewed by Thomas and Pritchard, 1987). Proteinases and peptidases of lactobacilli are not released into the medium but are bound to the cell wall. They may also act on substrates after lysis of the cells and release of cytoplasmic activities. The specificities of proteinases are usually determined on the basis of the site of their action on the various milk protein fractions. For example, the cell wall-associated proteinase of *L. helveticus* hydrolyzed both κ-casein and β-casein (Ezzat et al., 1985), and the enzyme from *L. bulgaricus* was active on all types of casein (Chandan et al., 1982). For further degradation, peptidases of various types are active: dipeptidase, tripeptidases, aminopeptidases, carboxypeptidase and arylamidases. The degradation products of proteins are transported into the cell by peptide transport systems, peptidase-coupled transport, and amino acid transport. Extracellular peptidase and transport systems of lactobacilli have been poorly characterized. Finally, intracellular peptidases degrade the peptides to amino acids. Generally, the proteinase and peptidase activities of thermophilic starter lactobacilli are higher than those of *S. thermophilus* (Shankar and Davies, 1978; Hemme et al., 1981). For the production of Emmenthal cheese, however, strains with low proteinase activity are required and are selected accordingly for use in starter cultures (Steffen, 1976; Steffen, 1979).

Antimicrobial Activities

Antagonistic activity of *Lactobacillus* cultures is a widely observed and frequently reported phenomenon. Early reports suggested the production of antibiotic-like substances by different lactobacilli (Wheater et al., 1951), *L. acidophilus* (Vincent et al., 1959; Sabine, 1963; Tramer, 1966; Fernandes et al., 1987), *L. helveticus* (Wheater et al., 1951; Vincent et al., 1959), and *L. lactis* (Wheater et al., 1952; Reiter et al., 1980). In part,

the antimicrobial effect could be related to the production of lactic acid (mainly responsible for pH reduction and the preservative effect in lactic fermented foods; Wood, 1985a) and of hydrogen peroxide, produced by *L. acidophilus* (Collins and Aramaki, 1980), *L. bulgaricus* and *L. lactis* (Dahiya and Speck, 1968), and shown to inhibit *Staphylococcus aureus* (Wheater et al., 1952; Dahiya and Speck, 1968) and *Pseudomonas* spp. (Price and Lee, 1970).

In vivo growth inhibition of *Escherichia coli* in the GI tract was attributed to the H_2O_2-induced activation of the lactoperoxidase antibacterial system of *L. lactis* (Reiter et al., 1980). This system has been reviewed by Reiter and Hänulv (1984). Although lactobacillin (an antibiotic-like substance of *L. lactis*) was shown to actually be H_2O_2 (Wheater et al., 1952), substances with typical antibiotic properties were indeed found to contribute to specific antimicrobial activities of lactobacilli. The role and relevance of natural antibiotics have only been studied in a few *Lactobacillus* strains, thus far. The bacteriocins have received special attention in recent years and have been reviewed by Daeschel (1989), Geis (1989), and Klaenhammer (1988). The present knowledge of bacteriocins of lactobacilli is summarized in Table 10. They can be clearly distinguished from other nonproteinaceous and broad-spectrum antibiotics of lactobacilli, and are defined as proteinaceous macromolecules that exert bactericidal activity against a limited range of organisms relatively closely related to the producer (Tagg et al., 1976). Factors such as specific bacteriocin receptors of sensitive cells and plasmids bearing genetic determinants for bacteriocin production and immunity are also involved (Daeschel, 1989). According to these criteria, only some of the previously mentioned natural antibiotics can be classified as bacteriocins. As a typical example, lactacin B from *L. acidophilus* inhibits a narrow spectrum of closely related organisms, such as *L. bulgaricus*, *L. helveticus*, *L. lactis* and *L. leichmannii* (Barefoot and Klaenhammer, 1984). Isolated as a thermostable macromolecular protein lipopolysaccharide complex of ca. 100 kDa (Barefoot and Klaenhammer, 1983), lactacin B was purified and an active protein component (6–6.500 kDa) that showed sensitivity to proteinase K was identified (Barefoot and Klaenhammer, 1984). The frequency of bacteriocin-producing strains appears to be high for some species, including *L. acidophilus*, which according to Barefoot and Klaenhammer (1983) is 63%, but these show inhibition only of closely related organisms. Mehta et al. (1983) reported a 5.4-kDa inhibitory protein from *L. acidophilus* AC$_1$ that showed a broad activity spectrum in vitro against various pathogenic organisms, including *Salmonella typhi*,

Table 10. Properties of bacteriocins from lactobacilli.

Bacteriocin type	Producer organism	Molecular weight (kDa)	Resistance to heat	Susceptibility to proteases	Maximum production (growth phase)	Inhibitory spectrum (susceptible organisms)	Evidence for plasmid involvement Production	Immunity	Plasmid size (MDa)	Reference
Lactacin B	L. acidophilus	6–6.5	+ (100°C, 60min)	+: protease, and proteinase K	Early stationary phase	Lactobacilli	–	–	–	Barefoot and Klaenhammer, 1983, 1984
Lactacin F	L. acidophilus	2.5	+ (99°C, 20min; (121°C, 15min)	+: ficin, protease K, trypsin, and B. subtilis protease	ND	Lactobacilli, and enterococci	+	+	68 and 52	Muriana and Klaenhammer, 1987, 1989
Unnamed	L. brevis	ND	+ (121°C, 60min)	+: pronase E, and trypsin	Late log phase	Lactobacilli, and leuconostocs	ND	ND	ND	Rammelsberg, 1988
Unnamed	L. casei	37–39	– (50°C, 90min)	–: pronase E, and trypsin	ND	Lactobacilli	ND	ND	ND	Rammelsberg, 1988
Unnamed	L. fermentum	ND	+ (96°C, 30min)	+: trypsin, and pepsin	ND	Lactobacilli	ND	ND	ND	DeKlerk and Smit, 1967
Helveticin J	L. helveticus	37	– (100°C, 30min)	+: trypsin, pepsin, ficin, proteinase K, pronase, and subtilisin	Late log phase	Lactobacilli	–	–	–	Joerger and Klaenhammer, 1986
Lactocin 27	L. helveticus	12	+ (100°C, 60min)	+: trypsin, and pronase, –: ficin	Early stationary phase	Lactobacilli	ND	ND	ND	Upreti and Hinsdill, 1975
Plantaricin A	L. plantarum	>8	+ (100°C, 30min)	+: protease	Mid-log phase	Lactic acid bacteria	–	–	–	Daeschel et al., 1986
Sakacin A	L. sakei	ND	+ (100°C, 20min)	+: trypsin, and pepsin	Late log phase	Lactobacilli, leuconostocs, enterococci, and listerias	+	+	18	Schillinger and Lücke, 1989

Symbols: +, present; –, absent; and ND, no data available.
Adapted from U. Schillinger, personal communication.

Shigella flexnerii, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The bacteriocinic nature of this substance has not been finally established. It appears that for typical bacteriocins from lactobacilli, antimicrobial activities have been reported only for closely related Gram-positive bacteria (Klaenhammer, 1988).

Several broad-spectrum antibiotic-like substances, such as acidophilin and lactocidin (from *L. acidophilus*) and bulgarican (from *L. bulgaricus*) are presumably not bacteriocins in nature (Vincent et al., 1959; Shahani et al., 1977; Reddy et al., 1984) but have not been chemically characterized. Showing broad-spectrum inhibition of nonrelated bacteria, and especially of Gram-negative intestinal pathogens, these antibiotics and their producer strains have been suggested to influence the microecology of the GI tract favorably (Shahani et al., 1977; Reddy et al., 1984; Fernandes et al., 1987; Whitt and Savage, 1987). Following an early suggestion by Winkelstein (cited by Vincent et al., 1959), the implantation of *L. acidophilus* and other intestinal lactobacilli in the digestive tract has received increased attention in recent years (Gilliland, 1979; Sandine, 1979; Shahani and Ayebo, 1980; Klaenhammer, 1982; Nahaishi, 1986). The suggested probiotic effects of certain lactobacilli in the GI tract may be brought about by a combination of different factors (including bacteriocins) referred to in this section.

In spite of increased research interest in bacteriocins and antibiotics of lactobacilli, relatively little is known about their in vivo activities in habitats such as the GI tract. Such information is important in view of the use of *Lactobacillus* preparations as dietary and therapeutic supplements and as protective cultures for food preservation. (Probiotics are discussed elsewhere in this section.) In vitro studies have shown that several factors influence the production and activity of bacteriocins. The bacteriocin-producing ability of some *L. acidophilus* strains was found to be related to the type of growth medium and was inhibited by bile salts (Shahani et al., 1976; Fernandes et al., 1988). Bactericidal activity of lactacin B from *L. acidophilus* (Barefoot and Klaenhammer, 1983) and plantacin B from *L. plantarum* (West and Warner, 1988) could only be demonstrated on solid media and not in liquid cultures. Optimal production of helveticin J (Joerger and Klaenhammer, 1986), plantaricin A (Daeschel et al., 1987), and sakacin A (Schillinger and Lücke, 1989) was observed during the mid- to late log phase of growth. For lactacin B (Barefoot and Klaenhammer, 1983) and lactocin 27 (Upreti and Hinsdill, 1975), however, maximal concentrations were detected during the early stationary phase. Bacteriocin production and its stability are likewise influenced by the initial pH of the growth

medium, and Muriana and Klaenhammer (1987) reported maximum yield of lactacin F when the pH of MRS broth was maintained at 7.0 during cultivation of *L. acidophilus* 88. Joerger and Klaenhammer (1986), however, observed the highest accumulation of helveticin J when *L. helveticus* was cultivated at pH 5.5. *Lactobacillus* bacteriocins generally show a bactericidal mode of action specifically against closely related organisms, excluding all Gram-negative bacteria (Klaenhammer, 1988; Daeschel, 1989). In contrast to the lactococci, the involvement of plasmid DNA could not be demonstrated for the majority of bacteriocins produced by lactobacilli, the exceptions being lactacin F (for *L. acidophilus*; Muriana and Klaenhammer, 1987) and sakacin A (for *L. sakei*; Schillinger and Lücke, 1989). Using electroporation and conjugation techniques, various plasmids have been transferred to *L. acidophilus* strain ADH, while transduction of plasmid DNA mediated by temperate bacteriophage (φ adh) could also be demonstrated (Luchansky et al., 1989). Reuterin, a potent, broad-spectrum antibiotic active against several Gram-positive and Gram-negative bacteria, fungi, yeasts and protozoa, is produced by *L. reuteri* when glycerol is present in the medium (Talarico et al., 1988). The antimicrobial agent was identified as an equilibrium mixture of "monomeric, hydrated monomeric, and cyclic dimeric forms of β-hydroxypropionaldehyde" (Talarico and Dobrogosz, 1989). The production of these and related substances does not appear to be restricted to *L. reuteri* but was also reported for *Streptococcus lactis* var. *maltigenes* by Morgan et al. (1966), and for *L. buchneri* and *L. brevis* by Schütz and Radler (1984). Being nonproteinaceous and showing extremely broad-spectrum antimicrobial activity, it cannot be classified as a bacteriocin. Results obtained from in vitro studies suggest that reuterin is effectively produced under pH, temperature, and relative anaerobic conditions resembling those in GI tract regions inhabited by *L. reuteri* (Chung et al., 1989; Dobrogosz et al., 1989). The presence of a variety of different microorganisms was shown to stimulate the heterologous production of reuterin (Chung et al., 1989), while several strains of lactobacilli inhabiting the GI tract were found insensitive to reuterin (Dobrogosz et al., 1989). These properties have been discussed with reference to a high potential of *L. reuteri* for use as a dietary adjunct, or even of purified reuterin for food preservation (Chung et al., 1989; Daeschel, 1989; Dobrogosz et al., 1989).

Genetics

Only limited data are available on the genetics of lactobacilli. With regard to their multiple

nutritional requirements, Morishita et al. (Morishita et al., 1974; Morishita et al., 1981) could show that the genes coding for the synthesis of most amino acids and vitamins are not absent but exist as silent genes. With *L. casei*, *L. plantarum*, *L. helveticus* and *L. acidophilus* at a frequency of 10^{-4} to 10^{-8} per survivor, mutants could be obtained which had reverted to prototrophy for an amino acid or vitamin. For *L. casei*, quintuple mutants were created which had lost the requirement for serine, aspartic acid, leucine, isoleucine, and tyrosine. Thus, the requirement for specific nutrients in lactobacilli may originate from some minor defects in a specific structural gene, the promotor, or a regulatory element. The number of the eliminated pathways and the degree of mutation leading to this effect depends on the environment to which a species is adapted (Morishita et al., 1981).

Lactobacilli contain plasmids, as was first shown by Chassy et al. (1976). Most are cryptic but the following metabolic functions have been found to be encoded by plasmids: lactose metabolism (Shimizu-Kadota, 1988; Chassy and Alpert, 1989), drug resistance (Gibson et al., 1979; Ishiwa and Iwata, 1980; Vescovo et al., 1982; Lin and Savage, 1986; Axelsson et al., 1988), maltose utilization (Liu et al., 1988), protein hydrolysis (Morelli et al., 1986), cysteine metabolism (Shay et al., 1988), fermentation of *N*-acetyl-D-glucosamine (Smiley and Fryder, 1978), and bacteriocin production and immunity (Muriana and Klaenhammer, 1987; Schillinger and Lücke, 1989).

Conjugation (Gibson et al., 1979; Vescovo et al., 1983; Shrago et al., 1986; Thompson and Collins, 1988b; Langella and Chopin, 1989) and transduction (Raya et al., 1989) have been shown to function as mechanisms of recombination in lactobacilli. Transfer of a nonconjugative vector by means of conjugative mobilization has been performed with *L. plantarum* (Shrago and Dobrogosz, 1988). No evidence for transformation or natural competence is available. On the other hand, in vitro transformation has been performed with protoplasts. This method yields low transfer rates only (Lin and Savage, 1986; Morelli et al., 1987). Higher transfer rates were obtained by electroporation (Chassy and Flickinger, 1987b). In addition, intrageneric (Iwata et al., 1986) and intergeneric (Cocconcelli et al., 1986) protoplast fusion and transfection (Shimizu-Kadota and Kudo, 1984a; Boizet et al., 1988; Cosby et al., 1988) have been successfully applied.

For genetic engineering experiments, *E. coli-Lactobacillus* shuttle vectors were constructed from endogenous plasmids of lactobacilli (Leer et al., 1987; Josson et al., 1989). Vectors derived from replicons of other Gram-positive organisms, mostly lactococci, have been constructed and successfully transferred into lactobacilli (Kok et al., 1984; de Vos, 1987). For stable maintenance of new properties, DNA can be integrated into the *Lactobacillus* chromosome (Scheirlinck et al., 1989). Transposons have also been introduced into lactobacilli (Aukrust and Nes, 1988; Knauf et al., 1989). Heterologous genes have been expressed in lactobacilli coding for α-amylase, endoglucanase (Bates et al., 1989; Scheirlinck et al., 1989), and antibiotic resistance.

Bacteriophages

Lactobacillus phages, which can be isolated from the common habitats of lactobacilli, were reviewed by Sechaud et al. (1988) and were also treated in the greater context of LAB by Josephsen and Neve (1998) and Brüssow (2001). The importance of these phages becomes obvious in food fermentations where they interfere with the process in a way that may result in downgrading of the product, in a time delay, or even complete breakdown of the fermentation process. As the use of starter cultures is most developed in the dairy field, the effect of phages is best known for lactococci. Phages of thermophilic lactobacilli have been found responsible for difficulties in acidification encountered in the preparation of cheese and yogurt (Peake and Stanley, 1978; Accolas and Spillmann, 1979). Virulent phages specific for *L. delbrueckii* subsp. *lactis* and subsp. *bulgaricus* and *L. helveticus*, respectively, used for the production of Swiss cheese or yogurt have been reported (Kiuru and Tybeck, 1955; Sozzi and Maret, 1975; Peake and Stanley, 1978; Alatossava and Pythilä1980; Trautwetter et al., 1986). In addition, *L. acidophilus* phages were detected in acidophilus milk (Kilic et al., 1996), and Watanabe et al. (1970) and Shimizu-Kadota and Sakurai (1982) described virulent phages specific for *L. casei*. The phages were isolated from yakult, which is a fermented milk used as a probiotic.

Despite the fact that the field of application is much broader than that of lactococci, no reports of phage problems in products such as fermented sausages, fermented vegetables, sourdough or silage are known. This can be explained by the fact that the fermentation of these products depends less on starters and takes place in a nearly solid matrix. In addition, it appears likely that in cases where the fermentation process was partially inhibited, phages might have been the causative agent but not looked for. There are few reports of studies of the interrelationship between phage and lactobacilli in meat products (Biewald, 1968; Trevors et al., 1983; Trevors et al., 1984; Nes and Sorheim, 1984; Heidel and Hammes, 1990; Leuschner et al., 1993). From

these studies it was concluded that phages are present in meat products or even in starter culture preparations, but they cause only a time delay in fermentations performed with model substrates. The same may apply to fermented cereals such as corn silage and sour dough, as phages have been isolated from these substrates specific for strains of *L. plantarum*, *L. fermentum* and *L. brevis*, respectively (Caso et al., 1995; Foschino et al., 1995; Ottogalli et al., 1996). Bacteriophages were also isolated from fermented vegetables, which were specific for strains of *Leuconostoc mesenteroides* and *L. plantarum* (Yoon et al., 2002). The authors observed that when low salt fermentation procedures were developed to reduce waste chloride production, starter cultures are needed for sauerkraut fermentation. It was argued that under these conditions phages may cause problems.

Reports of phage-carrying bacteria involved in the malolactic fermentation suggest a potential threat of bacteriophage to the wine industry. Lee (1978) demonstrated the existence of temperate phages in *L. casei* and *L. hilgardii* strains isolated from wine. Virulent phages from abnormal malolactic fermentations were also isolated, which were however active against *Oenococcus oeni* (Sozzi et al., 1982; Henick-Kling et al., 1986; Arendt and Hammes, 1992).

At the end of the lytic cycle, the newly synthesized phages are released from the host cell. Cell lysis depends on the activity of a lysin, which degrades the cell wall, and a holin, which forms lesions in the cell membrane. The bacteriophage PL1 lysin, purified from lysates of *L. casei* is a *N*-acetylmuramidase with a rather specific activity against the host (Watanabe et al., 1984a; Hayashida et al., 1987). The holin of *L. gasseri* phage adh lacks sequence homology with other known holins but shares the high hydrophobicity and a pair of potential transmembrane domains with other holins described (Henrich et al., 1995).

Lysogeny in lactobacilli was first demonstrated by Coetzee and de Klerk (1962) in two strains of *L. fermentum*. Several studies have shown that lysogeny is widespread among lactobacilli (Sakurai et al., 1970; Tohyama et al., 1972; Yokokura et al., 1974; Stetter, 1977; Shimizu-Kadota and Sakurai, 1982; McArthur and Barefoot, 1986; Mata et al., 1986; Cluzel et al., 1987; Raya et al., 1989; Kilic et al., 2001). Evidence for lysogeny was generally limited to detection of cell lysis following induction with mitomycin C or ultraviolet radiation and visualization of phage-like particles under the electron microscope. Plaque formation or lytic activity was detected only in a few cases in which indicator strains were available (Yokokura et al., 1974; Cluzel et al., 1987).

Although the most virulent phages are not genetically related to lysogenic phages (Klaenhammer and Fitzgerald, 1994), some temperate phages are a potential source of virulent phages, as it was shown by the example of *L. casei* phage (FSW). This temperate phage converts into a virulent phage (FSV) either by point mutation or by transposition of a mobile genetic element in the phage genome arising from the bacterial chromosome (Shimizu-Kadota et al., 1983; Shimizu-Kadota and Tsuchida, 1984a; Shimizu-Kadota et al., 1985). This mobile genetic element, called "ISL1," is the first transposable element described in lactobacilli.

Thus, the presence of temperate phages in a population may be a constant source of virulent phages and leads to problems in fermentation processes or, in a wider sense, to phage: fermenter associated problems. The latter was discussed by Kilic et al. (2001), who isolated temperate phages from vaginal strains of *L. crispatus*, *L. gasseri* and *L. jensenii*. The phages exhibited a broad host range and the authors concluded that phages may eliminate or repress vaginal lactobacilli, thereby contributing to vaginal bacteriosis. Temperate phages were also obtained from the human intestinal isolate *L. gasseri* adh, originally described as *L. acidophilus* (Raya et al., 1989).

Studies of Sozzi et al. (1976) suggest that MRS is the optimal medium for the formation of plaques by bacteriophages of thermophilic lactobacilli. The addition of calcium chloride sometimes favors the formation of plaques and is therefore recommended. LAB and their phages were found to have the same incubation temperature optima. Thus, temperature regulation cannot be used to prevent phage attacks in industrial fermentations (Sozzi et al., 1978). In fact, thermal resistance of the phages may exceed that of the hosts. Quiberoni et al. (1999) investigated the thermal resistance of *L. helveticus* phages and determined that 8.5–33 min at 63°C in MRS broth was needed for 99% inactivation. Bacteriophage-inactivation effects of basic amino acids (Murata et al., 1974), thiol reducing agents (Murata et al., 1972), and ascorbic acid (Murata et al., 1971) have been reported suggesting that the target attacked was not protein but DNA. Of the various chemical agents commonly used as biocides in industries and laboratories, sodium hypochlorite (100 ppm free chlorine residual) and peracetic acid (0.15 %) were found to be very effective. Quiberoni et al. (1999) showed that complete inactivation of all phages studied was achieved after a 5–10 min exposure.

All *Lactobacillus* phages investigated have double-stranded linear DNA. The replicated genomes are packaged by different mechanisms. The *L. delbrueckii* phages LL-H, mv4, *L. plan-*

tarum phage -g1e and *L. johnsonii* prophage Lj 965 have been found to contain sequences consistent with *pac*-site phages, while *L. gasseri* phage adh and *L. casei* phage A2 have sequences consistent with *cos*-site phages (Brüssow, 2001). The genome size ranges from 40 kbp found for the phage PL-1 of *L. casei* to 133 kbp found for the phage *fri* of *L. plantarum* (Nes et al., 1988; Caso et al., 1995). There may be a correlation between the large genome size of phage B2 and its small burst size of only 12–14 phages per cell. Phage PL-1 has a burst size of 200–600 phages per cell. Phage IL-1 of *L. casei* has also a genome size of 40 kbp and a burst size of 200 phages per cell.

Electron microscopic examination showed the tail-first orientation of phage adsorption (Watanabe et al., 1984b). Studies of receptor sites have shown that carbohydrate components in the cell wall of e.g., *L. casei* are used as initial phage receptors. Rhamnose seems to be essential, but additional carbohydrate moieties in the vicinity of the adsorption sites are also involved (e.g., galactose, glucose or their acetylated forms; Josephsen and Neve, 1998). Addition of rhamnose and galactose inactivated the phage, and a phage-resistant mutant lacked galactosamine in its surface component (Yokokura, 1977a). This effect is interesting, as this species of *Lactobacillus* has no teichoic acid in its cell wall, indicating that wall teichoic acid is not involved here in adsorption as it is in some other Gram-positive bacteria such as staphylococci.

Morphology of *Lactobacillus* phages was, in the absence of further information, the classification criterion used in accordance with the system of Bradley (1967). The phages are differentiated into three main classes based on tail morphology (i.e., morphotypes A [contractile tails], B [long noncontractile tails], and C [short noncontractile tails]) and further differentiated on the basis of head morphology (i.e., morphotype 1 [isometric], 2 [small prolate], and 3 [large prolate]). In modern phage taxonomy, the groups A–C are known as phage families Myoviridae, Siphoviridae and Podoviridae, respectively (Ackermann and DuBow, 1987). The *Lactobacillus* phages belong to the families Myoviridae and Siphoviridae (Josephsen and Neve, 1998).

The most valid criterion in studies of phage taxonomy is DNA homology, demonstrated by DNA-DNA hybridization. A close relatedness between virulent and temperate phages of lactobacilli has also been demonstrated by DNA similarity studies. Mata and Ritzenthaler (1988) and Lahbib-Mansais et al. (1988) compared 18 virulent and 4 temperate phages of *L. delbueckii* subsp. *bulgaricus* and subsp. *lactis* by DNA-DNA hybridization. All temperate and 15 virulent

phages could be alloted to similarity group a, and group b contained the remaining three virulent phages. Five virulent phages of *L. helveticus* were also investigated and were shown to be related to one another but unrelated to the phages of groups a and b. Cluzel et al. (1987) investigated 10 temperate and 16 virulent *L. delbrueckii* subsp. *bulgaricus* and subsp. *lactis* phages and could assign 7 temperate and 12 lytic phages to the previously described group a. Phages differing completely from those of groups a and b were assigned to two new unrelated groups called "c" and "d." The phages of groups a, c, and d share the same host range. Deeper insight in phage taxonomy became possible by the availability of sequencing data. These data exist for phages from five *Lactobacillus* species: *L. delbrueckii*, *L. gasseri*, *L. plantarum*, *L. casei* and *L. johnsoni* (Brüssow, 2001). No significant nucleotide sequence similarity was detected between *Lactobacillus* phages infecting distinct species (Desiere et al., 2000; Desiere et al., 2002). The overall genomic organization of *pac*-site phages was similar in *L. plantarum* phage phi-g1e, *L. delbrueckii* phage LL-H and *L. johnsonii* prophage Lj965. Vasala et al. (1993) demonstrated close relationships between the virulent and temperate *pac*-site *L. delbrueckii* phages LL-H and mv4, respectively.

The bacteriophage attack on LAB starter organisms is a permanent threat to industries relying on fermentation processes, and therefore much research has been devoted to phage resistance of starter cultures. The focus of the interest was on the characterization of different phage-resistance mechanisms in lactococci (reviewed by Coffey and Ross, 2002). However, little is known about natural mechanisms in lactobacilli (Moscoso and Suarez, 2000). Eguchi et al. (2000) described the abortive infection of *L. plantarum* as a plasmid-encoded mechanism of phage resistance. Alvarez et al. (1999) and Martin et al. (2000) introduced phage resistance into *L. casei* ATCC 393. The authors developed a transformation system and used it to integrate the phage repressor gene (*cI*) into the genome of the host. The modified strain fermented in the presence and absence of phages as well as adsorbed phage particles.

The Genus *Carnobacterium*

Certain atypical lactobacilli that were repeatedly isolated from refrigerated vacuum-packaged beef (Von Holy, 1983) were thought to be heterofermentative (Holzapfel and Gerber, 1983) and clearly differed from the atypical streptobacteria of meat and meat products described by Reuter (Reuter, 1970; Reuter, 1975). Because of the dif-

ferences from all other taxonomic groups and species of lactobacilli described to that stage, these bacteria were incorporated in a new species, *Lactobacillus divergens* (Holzapfel and Gerber, 1983). Properties such as CO_2 and L(+)-lactic acid production from glucose, and their relative inability to grow on acetate agar (Holzapfel and Gerber, 1983; Shaw and Harding, 1984; Shaw and Harding, 1985) indicated a relationship to atypical lactobacilli isolated from chicken meat (Thornley, 1957; Thornley and Sharpe, 1959; Barnes, 1976) and vacuum-packaged meat (Hitchener et al., 1982; Shaw and Harding, 1984). From the latter, another new species, *Lactobacillus carnis*, was isolated (Shaw and Harding, 1985), which however was found to be homologous to representatives of a salmonid fish pathogen (Cone, 1982), *Lactobacillus piscicola* (Hiu et al., 1984), with which *L. carnis* was reduced to synonymy (Collins et al., 1987). In an attempt to clarify their taxonomic position, Collins et al. (1987) applied physiological and biochemical criteria to classify these lactobacillus-like organisms into a new genus, *Carnobacterium*. The majority of the poultry strains were allocated to *Carnobacterium divergens* and *Carnobacterium piscicola*, while the remainder were incorporated into two new species, *Carnobacterium gallinarum* and *Carnobacterium mobile*. Fish was identified as a natural habitat of *C. divergens* and *C. piscicola* (Ringø and Gatesoupe, 1998a) as well as of a new species, *Carnobacterium inhibens* (Jöborn et al., 1999). Finally, *Carnobacterium alterfunditum* and *Carnobacterium funditum* (Franzmann et al., 1991) were isolated from an Antarctic anaerobic lake, showing that carnobacteria species inhabit also non-animal or non-food associated environments. The analysis of 16S rRNA relatedness (Collins et al., 1987) as well as phenetic studies (Lai and Manchester, 2000) revealed that *Lactobacillus maltaromicus* (Miller et al., 1974) is closely related to *Carnobacterium piscicola* (Collins et al., 1991), and therefore this organism will be treated herein as a *Carnobacterium*. In Fig. 12, the relatedness of the *Carnobacterium* species is depicted. The relatively far distance from the larger taxonomic unit containing lactobacilli is evident.

Habitats

Although distant from lactobacilli on the basis of 16S rRNA similarity, carnobacteria have a close phenotypic resemblance to atypical lactobacilli and comparable nutritional requirements, suggesting that carnobacteria and lactobacilli may share common habitats. This ecological relationship may also explain why carnobacteria, although isolated and studied before, were recognized as a separate taxon only recently.
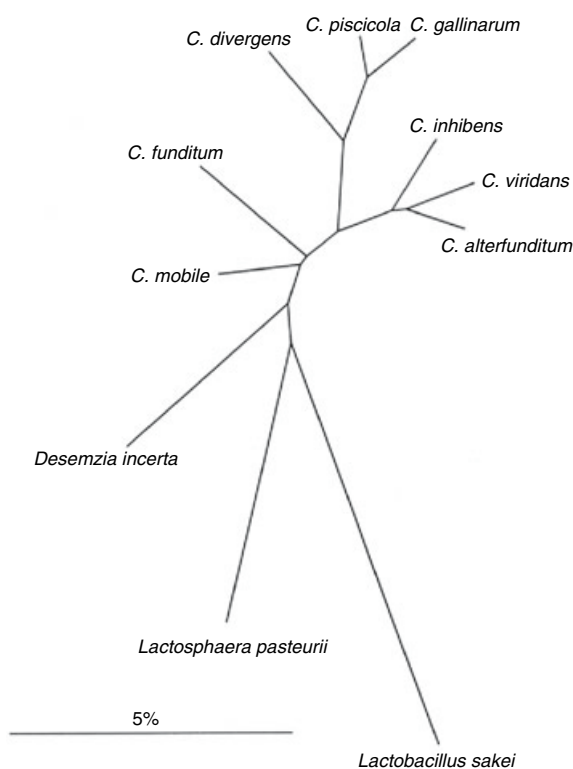
Fig. 12. Phylogenetic tree reflecting the relationships of *Carnobacterium* species. The tree was reconstructed applying the maximum parsimony analysis of all available at least 90% complete 16S rRNA sequences of Lactobacillaceae and carnobacteria. Alignment positions which share identical residues in at least 50% of all sequences of the depicted genera were considered. The positioning of *C. mobile* is based on partial sequence data and may be subject to changes. The bar indicates 5% estimated sequence divergence.

MEAT AND POULTRY The report of Thornley (1957) who isolated atypical lactobacilli from irradiated chicken meat, in which numbers of 10^8 cfu/g were reached during storage in a nitrogen atmosphere at 5°C, was probably the first on strains corresponding to carnobacteria. Mention was made of the failure of these strains to grow on acetate-containing media (Thornley, 1957). In a more detailed study of isolates from chicken and minced beef, Thornley and Sharpe (1959) identified three groups of atypical lactobacilli, all having diaminopimelic acid in their cell walls. Feresu and Jones (1988) included these strains in a comprehensive numerical taxonomic study and showed that group 2 of Thornley and Sharpe (1959) forms three clusters. Two of the clusters resembled *Lactobacillus carnis* (*C. piscicola*; phenon 9) and *L. divergens* (phenon 11), respectively. Collins et al. (1987) assigned the majority of the strains, originally isolated from chicken (Thornley, 1957; Thornley and Sharpe, 1959), to

these two species and to *Brochothrix thermosphacta*, while the remainder was assigned to two new species, *C. gallinarum* and *C. mobile*. One strain (NCDO 1230) identified as *C. piscicola* was isolated from human plasma (Collins et al., 1987).

The association of atypical lactobacilli (probably resembling carnobacteria) with meat and meat products has been mentioned by several workers. Among 140 strains of streptobacteria isolated from bacon, 11 failed to grow on acetate agar at pH 5.8 (Kitchell and Shaw, 1975). Reference to atypical streptobacteria as one of the major groups found in vacuum-packaged bacon was made by Tonge et al. (1964), Kitchell (1964), and Kitchell and Shaw (1975). This and other groups of lactobacilli reach numbers of 10^8 cfu/g in vacuum-packaged Wiltshire bacon, both under refrigeration and at room temperature. "Sour spoilage" resulting from their metabolic activities is not considered a less adverse defect than that caused by proteolytic bacteria. The real identity of these atypical streptobacteria remains undetermined. The majority of these strains isolated from bacon probably resembles *Lactobacillus curvatus* and *Lactobacillus sakei* and can be clearly distinguished from carnobacteria, which share some common phenetic properties not found among the lactobacilli (see Table 2).

Blickstad and Molin (1983) reported the isolation of unidentified lactobacilli from smoked pork loin and frankfurter sausages, and Borch and Molin (1988) identified *C. divergens* as one of the dominating psychrotolerant bacterial groups on cured, CO_2-packed pork and sliced, vacuum-packed ham. *Carnobacterium piscicola* was also isolated from fermented sausages (Schillinger and Lücke, 1987), which contrasts with its usual nonaciduric habitat (Shaw and Harding, 1984; Shaw and Harding, 1985).

The foremost and hitherto recognized man-made habitat of *C. divergens* and *C. piscicola* appears to be unprocessed, refrigerated red meat. Vacuum packaging of the specific nutrient-rich substrate and refrigeration seem to create a favorable ecosystem in which these carnobacteria may dominate. Thus far, these two *Carnobacterium* species have been isolated from minced meat (Holzapfel and Gerber, 1983; Shaw and Harding, 1984; Shaw and Harding, 1985), refrigerated vacuum-packaged unprocessed beef, pork and lamb (Hitchener et al., 1982; Shaw and Harding, 1984; Shaw and Harding, 1985; Schillinger and Lücke, 1986; Schillinger and Lücke, 1987; Borch and Molin, 1988), and CO_2-packed pork (Borch and Molin, 1988). In more recent microbiological studies of vacuum-packed beef, *Lactobacillus algidus* (Kato et al., 2000) and *L. fuchuensis* (Sakala et al., 2002) were detected and described as species. Mention was made that

these had been associated, among others, with *Carnobacterium divergens* and *C. piscicola*.

FISH *Carnobacterum piscicola* was first detected as a fish pathogen and isolated from diseased rainbow trout (*Salmo gairdneri*), cut throat trout (*Salmo clarki*), and chinook salmon (*Oncorhynchus tshawytscha*; Cone, 1982; Hiu et al., 1984). These bacteria have been isolated over several years from hatcheries in the State of Oregon and were most frequently associated with infected fish of one year and older, especially under conditions of stress associated with handling and spawning (Evelyn and McDermott, 1961; Ross and Toth, 1974; Hiu et al., 1984). The infections caused pathological lesions and blisters on and under the skin, and different signs in the internal organs. Thus far, *C. piscicola* exclusively has been found to cause septicemia (lactobacillosis) in salmonid and other fish species and to be associated with clinical and subclinical peritonitis in Australian salmonids (Humphrey et al., 1987).

At a later stage, it was observed that *Carnobacteria piscicola* is commonly associated with healthy fish. By contrast, this species was isolated from diseased, farmed fish such as rainbow trout (see above; Toranzo et al., 1993) as well as striped bass and catfish (Baya et al., 1991), suggesting that virulent strains may exist. There are, however, no virulence factors known and the strains did not exhibit hemolytic or phospholipolytic activity. Upon peritoneal infection with *C. piscicola*, an LD_{50} of $1–2 \times 10^6$ was determined for rainbow trout, whereas striped bass survived doses of $>10^8$. These values were found to be high in comparison to the infectivity of *Vibrio anquillarum* and *Aeromonas hydrophila* exhibiting LD_{50}-values in trout of 4.5×10^3 and 3.2×10^4, respectively. Remarkably, the isolated carnobacteria proved to be resistant to the chemotherapeutic agents widely used in aquaculture. Among 18 compounds, erythromycin was the most effective inhibitory compound.

A thorough investigation of the intestinal microflora of rainbow trout was performed by Spanggard et al. (2000). The studies included comparison of microscopic counts with plate counts, and a high percentage (>50%) of bacteria were found to be culturable. This recovery rate is remarkable, as the fish skin was found to contain <0.01% culturable bacteria only (Bernardsky and Rosenberg, 1992). The counts of the intestinal flora varied by 3–5 log units, and the ratio of Gram-negative/Gram-positive bacteria (mainly carnobacteria) varied greatly with the fish farm source. Out of a total of 120 isolates from trout of one farm, 43 were identified as carnobacteria, whereas no carnobacteria were found in fish from another farm. In a study of LAB associated with Atlantic salmon (Ringøet

al., 2000b), it was observed that among 752 isolates obtained from food, water and the digestive tract, 201 were identified as carnobacteria and 199 of these were isolated from the intestines.

In a review, LAB in fish were treated broadly by Ringø and Gatesoupe (1998a). The authors concluded that carnobacteria (in addition to streptococci, leuconostocs and lactobacilli) belong to but do not dominate the normal gastrointestinal flora of healthy fish. Their numbers are affected by nutritional and environmental factors such as dietary polyunsaturated fats (Ringø et al., 1998b) and stress (Ringø et al., 1997; Ringø et al., 2000b). The *Carnobacterium* species detected in the intestines were *C. divergens* in Arctic charr (*Salvelinus alpinus*; Ringø et al., 1997), Atlantic cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*), saithe (*Pollachius virens*; Strøm, 1988), wolffish (*Anarhichas lupus*; see Ringø and Gatesoupe, 1998a), and carp (*Cyprinus carpio*; Bausewein, 1988), *C. piscicola* in Arctic charr (Ringø et al., 1997), Atlantic salmon (see Ringø and Gatesoupe, 1998a), and rainbow trout (Starliper et al., 1992), and *C. inhibens* in Atlantic salmon (Jöborn et al., 1999).

Ringø and Holzapfel (2000a) showed that carnobacteria also inhabit the gills. Their numbers in Atlantic charr were approximately 3×10^4 cfu/g. Of the 100 isolates from the gills, 26 were attributed to carnobacteria and described as *C. piscicola*-like. Nine out of ten of these carnobacteria strains exhibited strong in-vitro growth inhibitory activity against the fish pathogens *Aeromonas salmonicida*, *Vibrio anguillarum* and *V. salmonicida*.

Carnobacterium piscicola and *C. divergens* belong also to the LAB population associated with seafood. Mauguin and Novel (1994) isolated 86 LAB from fresh Pollock, brine shrimp, gravad fish, vacuum packed seafood (surimi, smoked tuna, and salted cod), and fish stored under 100% CO_2 at 5°C (smoked tuna, fresh and salted cod, and salmon) and identified 16 strains as carnobacteria. DNA-DNA hybridization showed that 9 were *C. divergens* and 3 *C. piscicola*. Forty-five isolates were allotted to *Lactococcus lactis* subsp. *lactis*, 4 to *Lactobacillus plantarum*, and 8 to *Leuconostoc*.

The study of the microbial ecology of cold smoked salmon (Leroi et al., 1998) revealed that upon storage at 8°C in vacuum packages, Gram-negative bacteria prevailed for two weeks. The dominant LAB was *C. piscicola* (101 out of 155 LAB isolates). Between 2 and 5 weeks, lactobacilli were the prevailing LAB. These were found not to contribute to off odor, whereas the carnobacteria exhibited a weak potential to do so.

CHEESE Cheese was identified as a habitat of carnobacteria. A source was soft cheese made from nonpasteurized milk (Millière et al., 1994). It is assumed that these carnobacteria originate from the nonpasteurized milk upon contamination from the environment. Some isolates from Brie cheese of 5 dairy plants achieved 10^8–10^9 cfu/g, indicating a substantial potential to affect the property of the cheese. However, the effect of the carnobacteria on the sensory properties of cheese has not been studied. The pH of the cheese was 6.8–7.6 and thus quite favorable for carnobacteria to grow.

Carnobacteria were also detected in the curd obtained during Mozzarella cheese production. In that process, whey from the previous lot of cheese serves as inoculum. In the curd, LAB numbers of ca. 10^7 cfu/g have been detected. Ten LAB strains were differentiated, two of which were *C. divergens* and *C. piscicola* representing ca. 70% of the LAB population.

SEA WATER *Carnobacterium funditum* and *C. alterfunditum* have been isolated from Ace Lake, Antarctica (Franzmann et al., 1991). It is a meromictic lake whose salinity increases from 0.6% at the surface to 4.3% at the bottom depth of 24 m. The ratio of the major cations to chloride ions in the lake suggests it is of seawater origin. The lake is usually covered with ice for nine months and the temperature varies little (i.e., between 1 and 2°C). The total organic carbon at a 22-m depth was about 60 mg/liter. The carnobacteria isolates are adapted to that ecosystem but grow optimally at 22–23°C (not above 30°C) in 1.7% (*C. funditum*) or 0.6% (*C. alterfunditum*) sodium chloride of a mineral salts medium containing yeast extract and fructose (141 YF-medium). It is assumed that the carnobacteria play a role in the initial creation of a reduced environment and in providing electron donors for exploitation by sulfate-reducing bacteria.

A second habitat of carnobacteria was found in Lake Vanda, Antarctica (Bratina et al., 1998). On the basis of 16S rRNA analysis, the isolates were identified by the authors as carnobacteria. Their sequence similarity was determined by C. Hertel and W. P. Hammes (unpublished results), and 98.7 and 99.3% homology to *Desemzia incerta* (strain LV66) and *Carnobacterium inhibens* (LV62:W1), respectively, was calculated. These bacteria were found at a depth of 61 m, within 5 m of the water column above the oxic-anoxic interface. In that oxic zone, a submaximum peak in the concentration of Mn(II) had been determined. The organisms reduced MnO_2, and strain LV62:W1 exhibited this property even in an oxic incubation with and without added sodium azide. It was suggested that the reduction is mediated by a diffusible compound and that the release of adsorbed trace metals accompany-

ing the solubilization of manganese oxides might provide the carnobacteria with a source of nutrients adsorbed to their surface in an extremely oligotrophic environment.

Physiological Properties

The metabolism of carbohydrate corresponds basically to that of facultatively heterofermentative lactobacilli. Inasmuch as they use hexoses and pentoses, the organisms should possess the activities of aldolase and ketolase. Generally, hexoses are metabolized to L(+) lactate, but CO_2, acetate and ethanol are also formed and, depending on the access of oxygen, formic acid and acetoin are produced in varying amounts. Formate formation requires anaerobic conditions (Borch and Molin, 1989). In *C. funditum* and *C. alterfunditum*, glycerol is fermented to mainly ethanol, acetic acid, and formic acid (Franzmann et al., 1991). With the exception of the latter species, the carnobacteria produce ammonia and citrulline from arginine (Montel et al., 1991).

The formation of biogenic amines is of concern in food-associated microorganisms. Studies performed by Leisner et al. (1994) and Jørgensen et al. (2000) showed that strains of *C. piscicola* as well as *C. divergens* have the potential to form tyramine but not histamine or phenylethylamine under refrigeration conditions (5°C).

Numerous principles contribute to the antagonistic potential of LAB (Hammes and Tichaczek, 1994; Holzapfel et al., 1995). Among these, bacteriocins from Groups I and II LAB (Klaenhammer, 1993; Nes et al., 1996) are commonly found and have been well studied. Also bacteriocinogenic carnobacteria are well known. An example of a class I compound (lantibiotics) is carnocin UI 49, which is formed by a *C. piscicola* strain isolated from fish (Stoffels et al., 1992a; Stoffels et al., 1992b), and examples of class II bacteriocins produced by carnobacteria isolated from fish, meat and cheese are numerous. The potential of carnobacteria to act antagonistically on competitors is comparably high and a major part of the active principles can be attributed to bacteriocins. For example, isolates identified as being *C. piscicola*-like, associated with the digestive tract of Atlantic salmon (*Salmo salar* L.), were screened for the ability to produce growth inhibitory compounds active against the fish pathogen *Aeromonas salmonicida*. It was observed that within 199 *C. piscicola*-like isolates 139 inhibited growth of the pathogen (Ringø et al., 2000b). Furthermore, among 33 isolates from French Brie cheese, 16 produced antimicrobial substances, which were most likely bacteriocins. One of these (Carnocin CP5) formed by *C. piscicola* CP5 was characterized in more detail (Herbin et al., 1997). The literature on bacteriocins produced by carnobacteria has been reviewed by Stiles (1994) and is also included in a general overview on class II bacteriocins (Ennahar et al., 2000). It can be concluded that the characteristics, genetics, membrane transport, and regulation of these compounds are similar to the general pattern of bacteriocins produced by LAB. From the perspective of food hygiene, it is of interest that carnobacteria share their man-made habitats (meat, fish products and cheese) with listeria. The occurrence therein of *L. monocytogenes* has been demonstrated and is of great concern (Farber and Peterkin, 1991). It is likely that the presence of carnobacteria in a "spoilage population" may have contributed traditionally to food safety, as the bacteriocins are especially effective against listeria. Several studies have been performed to apply carnobacteria as well as other LAB in protective cultures or as producers of biopreservatives (Stiles, 1996). Bacteriocins as the active principle may be used as preparations or formed in situ. Up to now, no commercial practical application of carnobacteria in either principle is known.

Isolation

Apart from their nonaciduric nature, carnobacteria associated with food of animal origin (including fish) possess the relatively complex nutritional requirements of the lactobacilli. Hiu et al. (1984) have found that *C. piscicola* isolates from diseased fish require folic acid, riboflavin, pantothenate, and niacin for growth, but not vitamin B_{12}, biotin, thiamine, or pyridoxal. *Carnobacterum divergens* and *C. piscicola* isolates from meat also do not require thiamine (De Bruyn, 1987a). A neutral to high pH of 6.8–9.0 favors growth, and pH 8.0–9.0 may serve to selectively inhibit lactobacilli that are often found in association with carnobacteria. The omission of acetate from conventional *Lactobacillus* media such as MRS (de Man et al., 1960) also favors the growth of carnobacteria. Less fastidious in their nutrition requirements are *C. alterfunditum* and *C. funditum* (Franzmann et al., 1991). These species grow on mineral salts, a carbohydrate source, and yeast extract, which cannot be substituted for by vitamin supplementation.

NONSELECTIVE AND SEMISELECTIVE ISOLATION When the microbial population is dominated by carnobacteria (which may be the case for vacuum packaged, refrigerated meat, and poultry, and for diseased fresh water fish), isolation is possible by direct streaking or plating onto some nonselective "universal" media. *Carnobacterium piscicola* was also found in vacuum-packaged cold-smoked salmon during storage at 8°C (Leroi et al., 1998).

From diseased fish, direct streakings were made onto brain heart infusion agar or tryptic soy agar (Hiu et al., 1984) that are commercially available. For the nonselective recovery of carnobacteria from refrigerated, vacuum-packaged meat, Standard-I-Agar (pH 7.2–7.5; Merck) has been found to be useful (Von Holy, 1983); aerobic incubation was either at 25°C for 3 days or at 7°C for 10 days. Alternatively, CASO-medium (Merck) or tryptic soy agar (Difco) with 0.3% added yeast extract may be used for this purpose.

In comparative studies with all carnobacteria species, it was shown that all grow in the following complex media: BHI (Lai and Manchester, 2000), tryptic soy medium supplemented with 2% NaCl (TSAS; Jöorn et al., 1999), and tryptic soy medium with added 5% glucose (Ringøet al., 2000b).

After adjusting the pH to 8.0 and substituting sucrose for glucose, Standard-I-Agar may be applied for the semiselective enumeration of carnobacteria in the presence of a dominating *Lactobacillus* population. The identity of typical catalase-negative colonies, however, should be verified by microscopy and use of some of the criteria in Table 2. It should be clear that no heme source is included in the media, as it was reported by Ringøet al. (2002) that all type strains supplied with heme exhibit catalase activity, probably by production of the heme free apoenzyme which becomes an active enzyme upon integration of the heme component.

Borch and Molin (1988) used tryptone glucose extract agar (TGE, Oxoid; pH 7.0) for determining the total bacterial count of refrigerated, prepacked meat and meat products and recovered *C. piscicola* and *C. divergens* after incubation at 28°C for 3 days.

In their studies on LAB in seafood, Mauguin and Novel (1994) used a fish extract medium (FEG) to isolate and to grow carnobacteria, which contains the following (g/liter): fish extract, 20; NaCl, 1; and glucose, 0.2. The fish extract was prepared from minced Pollock fillets mixed with distilled water (ratio 1:5). The mixture was centrifuged (10 min, 11,000 × γ) and the supernatant subjected to ultrafiltration (polysulfone filter membrane; Minitan system, Millipore; limit MW 10,000). The extract was lyophilized and stored at 5°C.

Prior to their recognition as a separate group, the carnobacteria were probably most frequently isolated on MRS and APT agar as part of the *Lactobacillus* population of meat and poultry (Thornley and Sharpe, 1959; Hitchener et al., 1982; Shaw and Harding, 1984; Schillinger and Lücke, 1986; Schillinger and Lücke, 1987). Since MRS agar was found to inhibit the growth of some *L. divergens*-isolates, it was modified (as "D-MRS") by increasing the pH to 8.5, omitting acetate, and by substituting sucrose for glucose (Hammes et al., 1992). The substitution by sucrose was necessary to reduce Maillard reactions during autoclaving. However, the studies of Lai and Manchester (2000) have shown that within Cluster B which comprises *C. divergens*, there are strains that do not ferment sucrose, and Franzmann et al. (1991) reported that *C. funditum* and *C. alterfunditum* do not grow on MRS without acetate.

D-MRS for Cultivation and Semiselective Enumeration of *Carnobacteria*

Universal peptone	10 g
Yeast extract	5 g
Beef extract	5 g
Sucrose	20 g
Tween 80	1 g
K$_2$HPO$_4$	2 g
Triammonium citrate	2 g
MgSO$_4$ ·7H$_2$O	0.1 g
MnSO$_4$ ·4H$_2$O	0.05 g

Dissolve ingredients in 1 liter of distilled water. For preparation of the solid medium, use 1.5% agar. Adjust pH to 8.5 using NaOH, before sterilizing at 121°C for 10–15 min. Increasing the MnSO$_4$ ·4H$_2$O concentration to 0.4% may stimulate colony growth of *C. divergens* and *C. piscicola* strains (Bosch and W. H. Holzapfel, unpublished observations). Incubate at 25°C for 3 days, either aerobically or using a reduced gas atmosphere (e.g., Anaerocult, Merck).

SELECTIVE ISOLATION MEDIUM There is only one selective medium known that can be used to isolate and enumerate *C. divergens* and *C. piscicola*.

Selective Cresol Red Thallium Acetate Sucrose (CTAS) agar (WPCM, 1989)

Peptone from casein	10.0 g
Yeast extract	10.0 g
Sucrose	20.0 g
Tween 80	1.0 g
Sodium citrate	15.0 g
MnSO$_4$ ·4H$_2$O	4.0 g
K$_2$HPO$_4$	2.0 g
Thallium acetate	1.0 g
Nalidixic acid	0.04 g
Cresol red	0.004 g
Triphenyl tetrazolium chloride	0.01 g
Agar	15.0 g
Water	1 liter

Dissolve ingredients in distilled water. Substitute 2% inulin for sucrose to distinguish *C. piscicola* colonies from enterococci. The latter may grow on CTAS medium but do not ferment inulin. In general, *C. divergens* shows sparser growth than *C. piscicola* and may appear as pinpoint sized colonies, often without changing the red color of the medium to yellow and showing a metallic-bronze shine.

Typical colony morphology, being convex, shiny, and ranging from yellowish to pinkish, can be best observed on CTAS agar when triphenyl tetrazolium chloride is omitted. When sucrose is substituted by inosine, *C. piscicola* strains espe-

cially show dry, umbonate colony growth comparable to "β-colonies" (Mitsuoka, 1969) often observed for *Lactobacillus acidophilus*.

The colonies of *C. inhibens* appear as semi-translucent when grown under anaerobic conditions and whitish when grown aerobically.

Cultivation, Maintenance, and Conservation

CULTIVATION Although MRS (broth or agar) can be used for the cultivation of axenic cultures, growth is often poor, while some strains are unable to sustain growth on subcultivation on this medium. APT medium (Evans and Niven, 1951) at pH 7.0, CASO-medium (Merck) with 0.3% yeast extract, or even Standard-I-Medium (Merck) at pH 7.2 provide better growth for practically all *Carnobacterium* strains. For general cultivation purposes, and especially for fastidious strains, D-MRS (pH 8.0–8.5) is recommended. Incubation is at 25°C for 24–48 h, either in air or a slightly reduced atmosphere.

MAINTENANCE General maintenance of cultures is as for lactobacilli, taking into account the non-aciduric nature of the carnobacteria and selecting APT, CASO, or D-MRS media in the pH range 7.0–8.5. Stab cultures in D-MRS agar (pH 8.0–8.5) should be kept at 1–4°C and transferred every 2–3 weeks. Addition of 5% calcium carbonate to cultivation broth (e.g., APT) may serve to protect vitality of stock cultures over several weeks at 1–4°C.

CONSERVATION Lyophilization as used for lactobacilli gives satisfactory results, provided cryoprotective agents (milk solids, lactose, or horse serum) are added to the cell suspension, and ampoules are sealed under high vacuum and stored at 8–12°C. Superior results are obtained by cryopreservation at –80°C using glycerol-peptone protective broth for suspending late logarithmic cells, harvested by centrifugation or by rinsing surface growth from agar media. Borch and Molin (1988) recommended the storage of strains as dense cultures in APT broth at –20°C.

Identification

The general description of the carnobacteria corresponds to that of the lactobacilli, insofar as the former are Gram-positive, catalase-negative, nonsporeforming rods, do not reduce nitrate, and have a fermentative metabolism. Three species are motile (Collins et al., 1987; Schillinger and Holzapfel, 1995). More precisely, the carnobacteria may be described as short to medium length, straight, slender rods with rounded ends, occurring singly, in pairs, or sometimes in short chains. Initially described as atypical heterofer-

menters (Holzapfel and Gerber, 1983), metabolic studies on *L. divergens* (syn. *C. divergens* DSM 20623) have shown that this organism ferments glucose via the glycolytic pathway (De Bruyn et al., 1987b; De Bruyn et al., 1988). Only some secondary decarboxylation/dissimilation of pyruvate/lactate to acetate, formate, and CO_2 was observed, explaining the slow and often minimal production of CO_2 in glucose broth media. Using D-[U-^{14}C] glucose, De Bruyn et al. (1988) showed that about 75% of the lactate and less than 10% of each of the formate and acetate were produced from glucose. It was postulated that the remainder of these products may be derived from endogenous non-glucose sources. Lactate and small amounts of ethanol and acetate are produced from ribose (Holzapfel and Gerber, 1983). Other properties that are shared with most lactobacilli include the inability to produce gelatinase, urease, indole or H_2S. The peptidoglycan is of the *meso*-diaminopimelic acid-direct type (described by Schleifer and Kandler, 1972). More than 95% of the lactic acid is produced as the L(+) isomer. Most strains grow at 0–2°C, especially in typical meat vacuum-packaged meats. No growth occurs at 45°C, in presence of 8% NaCl, or at pH 3.9 (Holzapfel and Gerber, 1983; Collins et al., 1987).

Using D-MRS broth (W. H. Holzapfel and Long, unpublished observations), optimum growth of *C. divergens* was observed within a pH of 8.0–9.5. Addition of 1.5% acetate raised the minimum pH for growth from about 4.8 to 6.0.

As shown by Collins et al. (1987) and recently confirmed by Ringøet al. (2002), oleic acid (Δ9,10) is present in the cellular fatty acids of all carnobacteria species. A group- or even species-specific pattern of the composition of fatty acids can be recognized. Oleic acid is the predominant compound in *C. piscicola* (*L. maltaromicus*), *C. gallinarum*, *C. mobilis* and *C. divergens*. The latter species contains also dihydrosterculic acid (9,10-methylenoctadecanoate). This cyclopropane derivative is biosynthetically related to oleic acid, and when counted together with that fatty acid, the above-mentioned species contain ca. 40–50% $C_{18:1}$ compounds. They also contain substantial amounts of $C_{16:1}$ fatty acids (ca. 13–23%). On the other hand, in the species *C. funditum*, *C. alterfunditum* and *C. inhibens*, C_{16}-fatty acids are predominant (ca. 50%). In *C. funditum* and *C. alterfunditum*, characteristically $C_{16:1}$ (Δ7,8), but no (Δ9,10) is detected, whereas in *C. inhibens* the reverse is true. In the former two species, a characteristic cyclopropane derivative (cyclo 20:1, 0.6 and 1.6 %, respectively) has been identified. The composition of the unsaturated fatty acids is consistent with the 16S rRNA data that indicates a closer relationship between the latter three species. *Carnobacterium mobilis* is

included in that branch but is high in $C_{18:1}$ and intermediary in the $C_{16:1}$ content.

The major properties for differentiating the carnobacteria from the lactobacilli are shown in Table 2. During isolation and identification procedures, the acetate sensitivity, as can be demonstrated by failure to grow on acetate agar (Rogosa et al., 1951), and the ability to grow at pH 8.5–9.0, may serve as routine tests for the recognition of carnobacteria among the rod-shaped LAB.

Differentiation of Species

Seven species are presently recognized to comprise the genus *Carnobacterium*: *C. divergens*, *C. gallinarum*, *C. mobile*, *C. piscicola* (Collins et al., 1987), *C. funditum*, *C. alterfunditum* (Franzmann. et al., 1991), and *C. inhibens* (Jöborn et al., 1999). Although these species represent seven separate DNA-DNA similarity groups, they have been found to belong to the same ribosomal RNA homology cluster (Collins et al., 1987; Champomier et al., 1989; Franzmann et al., 1991; Jöborn et al., 1999). Genomic data resting on sequence analysis of 16S rRNA permit a reliable differentiation of all *Carnobacterium*

species and may even be, together with other genotypic methods, the best way to differentiate phenotypically very similar species such as *C. piscicola* and *C. divergens*. The availability of the 16S rRNA sequences permits also the identification of diagnostic regions that can be used to construct species-specific probes or primers. These have been first employed by Brooks et al. (1992) to amplify 16S rRNA followed by oligonucleotide probes to identify *C. divergens*, *C. mobile* and *C. piscicola*/*C. gallinarum* at species level. The technique was applicable with purified DNA extracts, crude cell lysates, and food samples. Ringø et al. (2002) employed the amplified fragment polymorphism technique (AFLP(tm)) to characterize a *C. divergens* strain isolated from Arctic charr. The high resolving power of this technique showed that this isolate can be identified on the strain level and groups in AFLP cluster rather distantly from the type strain of the species.

Except for using the composition of the cellular fatty acids (see above), classical biochemical and physiological tools are only of limited value. In Table 11, the key physiological criteria for their differentiation are given. Simple physiological tests may be relied on for species differenti-

Table 11. Selected physiological characteristics of species of Carnobacterium.

Characteristic	*C. alterfunditum*	*C. divergens*	*C. funditum*	*C. gallinarum*	*C. inhibens*	*C. mobile*	*C. piscicola*
Growth at (°C)							
0	+	+	+	+	+	+	+
30	−	+	−	ND	+	+	+
40	−(+[b])	+	−(+[b])	ND	−	ND	+
Motility	+	−	+	−	+	+	−
Voges-Proskauer test	ND	+	ND	+	ND	−	+
Acid produced from							
Glycerol	w	ND	w	ND	−	+/−	+
Xylose	−	−	−	+	−	−	−
Galactose	w	−	w	+	+	+	+
Mannitol	−	−	+	−	+	−	+
Methyl D-glucoside	−[c]	−	−[c]	+	−	−	+
Amygdalin	+	+	−	+	+	−	+
Lactose	−	−	−	+	w	−	−
Melibiose	−	−	−	−	−	−	+
Trehalose	−	+	+	+	+	+	+
Inulin	−	−	−	−	w	+	+
Melezitose	−	+/−	−	+	−	−	+/−
Gluconate	−[c]	+	−[c]	+	−	−	+
D-Tagatose	ND	−	ND	+	−	−/+	−
D-Turanose	−[c]	−	−[c]	+	−	−	+/−
Hydrolysis of Esculin	−	ND	−	+	+	+	+
G+C content (mol%)	33–34	33.0–36.4	32–34	34.3–36.4	ND	35.5–37.2	33.7–36.4

Symbols: +, present; −, absent; w, weak; ND, no data.

[a]All species produce acid from cellobiose, fructose, glucose, maltose, mannose, ribose, salicin and sucrose, but not from adonitol, dulcitol, arabinose, glycogen, inositol, raffinose, rhamnose, and sorbitol.

[b]According to Franzmann et al. (1991), sucrose fermentation by *C. funditum* and *C. alterfunditum* is + and weak, respectively, whereas Lai and Manchester (2000) report − for both species.

[c]According to Lai and Manchester (2000). See text.

ation and are described by Borch and Molin (1988), Collins et al. (1987), Holzapfel and Gerber (1983), Shaw and Harding (1985), Montel et al. (1991), and Ringø et al. (2002). Sugar fermentation patterns (see Table 11) can especially be used for routine differentiation of *Carnobacterium* species. The key fermentable substrates for this purpose are amygdalin, arabinose, galactose, gluconate, inulin, lactose, mannitol, melezitose, melibiose, α-methyl-D-glucoside, ribose, tagatose, trehalose, D-turanose and D-xylose as well as glycerol. The API 10E and API 50 CH systems (Biomerieux), as applied by Collins et al. (1987), or the Minitek System (BBL; Borch and Molin, 1988), simplify the working procedure. These commercialized rapid test systems seem to produce similar results to conventional sugar fermentation tests in the broth used by Holzapfel and Gerber (1983) or on the agar as used by Shaw and Harding (1985). Shaw and Harding (1985) added 0.5% of a filter-sterilized sugar solution to the following basal agar medium after sterilization: yeast extract (Difco), 0.6%; trypticase (BBL), 1.5%; cysteine HCl, 0.02%; Tween 80, 0.1%; chlorophenol red, 0.004%; $MgSO_4 \cdot 7H_2O$, 0.02%; $MnSO_4 \cdot 4H_2O$, 0.005%; and agar, 1.2%; pH 6.4. Lai and Manchester (2000) used in their investigation of acid production from carbohydrates the following basal medium (SBM): peptone, 1% (w/v); NaCl, 0.5% (w/v); yeast extract, 0.1% (w/v); agar, 1.5% (w/v); and bromocresol purple, 15 ml liter^{-1} of 0.2% (w/v). This medium was supplemented with 0.5% (w/v) sterilized carbon source. The results were read upon incubation for 5 days at 25°C.

Taking into account the nonaciduric nature of the carnobacteria, adjustment of the pH of basal media to neutral and even higher may be advisable. Care should be taken that a suitable indicator of the adjusted pH value (e.g., bromocresol purple or cresol red) is chosen. Most tests used for lactobacilli (described elsewhere in this chapter) may be applied to the carnobacteria, provided a higher pH is used. For motility testing, stab inoculation into YGPB medium, modified to contain 0.1% glucose, 0.1% lactose, and 0.2% agar (Oxoid no. 3; Collins et al., 1987) is recommended.

All *Carnobacterium* strains seem to share the following common properties (Collins et al., 1987; Borch and Molin, 1988): acid production from cellobiose, D-fructose, D-glucose, maltose, D-mannose, salicin and sucrose (see comment in Table 11); no acid production from arabitol, dulcitol, erythritol, fucose, glycogen, inositol, raffinose, L-rhamnose, L-sorbose and xylitol. All strains investigated by Collins et al. (1987) produced arginine deiminase, and Lai and Manchester (2000) observed that all strains cleaved 4MU-β-D-glucopyranoside, grew in the presence

of potassium tellurite (0.1%, w/v) and colistin sulfate (10 κg/ml) and at pH 7.0. None of the strains grew in the presence of 10% (w/v) sodium chloride or produced acid from sodium pyruvate (0.1%, w/v).

Carnobacteria appear to be facultative anaerobes, as are most lactobacilli. Although cultivation in air does not seem to reduce colony growth of most strains on agar plate surfaces significantly, *C. divergens* strains especially show more prolific growth in a reducing environment. The recovery of inoculated cells of *C. funditum* and *C. alterfunditum* on agar is poor under aerobic conditions (Franzmann et al., 1991). Under anaerobic conditions *C. inhibens* cultures grow to higher cell density than in aerated cultures (Jöborn et al., 1999). Their relatively high resistance to thallium acetate and sodium azide enables most carnobacteria to grow well on media selective for *Enterococcus*, such as Barnes agar (Barnes, 1959) and CATC agar (Burkwall and Hartman, 1964; Reuter, 1968). *Carnobacterium piscicola* grows slightly better on these media than *C. divergens*, but their colonies appear relatively similar to those of typical enterococci. Investigations into the construction of selective media for carnobacteria revealed their resistance to heavy metals is more or less similar to that of strains of *Enterococcus faecium* and *E. faecalis* (Bosch and W. H. Holzapfel, unpublished observations).

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CHAPTER 1.2.11

Listeria monocytogenes and the Genus *Listeria*

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Introduction

The genus *Listeria* contains six species, two of which are pathogenic: *Listeria monocytogenes*, the food-borne human pathogen responsible for listeriosis, on which this chapter is focused, and *L. ivanovii*, an animal pathogen. Listeriae are Gram-positive rod-shaped bacteria with low G+C content, which are found in a variety of animals and niches, including processed food. They are resistant to extreme conditions, such as low temperature or high salt, demonstrating a great adaptability to their environment (Vazquez-Boland et al., 2001).

Listeria monocytogenes infections cause gastroenteritis, meningitis, neuro-encephalitis, chorioamnionitis, abortions, and neonatal infections. Listeriosis is associated with a high mortality rate, particularly in immunocompromized individuals (Schlech, 2000). In addition to its medical importance, *L. monocytogenes* contamination of food products raises important economic issues in the food industry.

Listeria monocytogenes is a facultative intracellular pathogen. It has evolved a panoply of virulence factors, which exploit important cellular processes during infection (Cossart et al., 2003; Cossart and Sansonetti, 2004). *Listeria monocytogenes* has emerged as a genetically manipulable tool to address key cell biology processes, such as phagocytosis, actin-based motility and signaling through growth factor receptors (Cameron et al., 2000; Cossart and Bierne, 2001; Bierne and Cossart, 2002a). Moreover, as a result of its intracellular life, *L. monocytogenes* mediates a strong T-cell response and is widely used as a model to study the CD8-mediated immunity of intracellular parasites (Edelson and Unanue, 2000; Badovinac et al., 2003; Lara-Tejero and Pamer, 2004). The detailed mechanisms of the immune response to *L. monocytogenes* will not be treated in this review, as they are elegantly and largely reviewed (Edelson and Unanue, 2000; Unanue, 2002; Badovinac et al., 2003; Lara-Tejero and Pamer, 2004, Pamer, 2004). The combined use of genetics, cell biology, functional genomics and post-sequencing studies has led to a precise understanding of *L. monocytogenes* infections. Comparative genomics of *L. monocytogenes* with the nonpathogenic species *L. innocua*, and other *Listeria* species can now be fully exploited for the study of virulence, regulation and biodiversity of Listeriae (Glaser et al., 2001; Cabanes et al., 2002; Buchrieser et al., 2003; Doumith et al., 2004a).

The Discovery of Listeriosis and Listeriae

Listeriosis was first described in the late 1920s and proposed to be contracted through oral contamination. This hypothesis was confirmed in the 1960s. A series of outbreaks in industrialized countries during the 1970s and 1980s definitely established that *L. monocytogenes* was indeed responsible for food-borne listeriosis (Schlech, 1984; Rocourt and Cossart, 1997).

The first official strain of the human pathogen *L. monocytogenes* was isolated in 1924 after an animal carehouse outbreak among rabbits and guinea pigs exhibiting severe mononucleosis (Murray et al., 1926). A clinical isolate of *L. monocytogenes* from a case of human meningitis was deposited at the Institut Pasteur in 1921 (Dumont and Cotoni, 1921). The first strain of the animal pathogen, *L. ivanovii*, was identified in the 1960s from lambs with congenital listeriosis (Ivanov, 1962).

Classification

Listeria spp. are small Gram-positive, nonspore forming, noncapsulated bacilli, generally motile at low temperatures (20°C), although some *Listeria* strains are nonmotile at 37°C because of lack of flagellin expression at this temperature (Way et al., 2004). Their DNA is characterized by a low G+C content (36–42%). They are related to *Clostridium*, *Bacillus*, *Enterococcus*, *Streptococcus* and *Staphylococcus* (Seeliger and Jones, 1986; Schmid et al., 2005). On the basis of DNA-

DNA hybridization, multi-locus enzyme electrophoresis (MEE), rRNA gene restriction patterns, and 16S rRNA sequencing, the genus *Listeria* comprises six species and two subspecies: *L. monocytogenes*, *L. ivanovii* subsp. *ivanovii*, *L. ivanovii* subsp. *londoniensis*, *L. seeligeri*, *L. innocua*, *L. welshimeri* and *L. grayi*. *Listeria monocytogenes* is a human and animal pathogen, and *L. ivanovii* is pathogenic for animals, mainly sheep and cattle. On rare occasions, *L. ivanovii* and *L. seeligeri* were shown to be associated with human infections (Rocourt and Cossart, 1997; Schmid et al., 2005).

Isolation and Characterization

Reservoirs

Listeriae are found in a wide variety of reservoirs ranging from soil, rotting plants, water, cattle milk and food to numerous animal species and humans. There is an asymptomatic carriage of *L. monocytogenes* in the intestinal tract (Seeliger and Jones, 1986; Grif et al., 2001). Listeriae can also colonize various inert surfaces and can form biofilms on food-processing surfaces (Roberts and Wiedman, 2003).

Detection and Isolation

Isolation of *Listeria* from contaminated or infected material can be obtained by direct plating on tryptic soy base agar supplemented with blood for human samples, but an enrichment step followed by plating on selective media is recommended for polycontaminated samples, such as food products. Enrichment procedures vary but are generally based on a one- or two-step enrichment in liquid selective broth, followed by plating on selective solid media, which allow identification of Listeriae (LPM, Oxford, or PALCAM agar), or specific identification of *L. monocytogenes* strains (Rapid L. Mono, ALOA [Agar *Listeria* Ottavani and Agosti], and Chromagar) (Figs. 1 and 2). Rapid detection of Listeriae in food isolates, for example, can be performed using immunoassay-based kits. However, PCR (polymerase chain reaction) is the technique of choice for rapid detection of *L. monocytogenes* in both clinical and food isolates. Isolation remains indispensable for epidemiological studies (Swaminathan et al., 1995; Allerberger, 2003).

Identification

Genus identification is based on classical tests including Gram staining, observation of motility, and biochemical reactions, such as catalase and acid production from D-glucose (Swaminathan
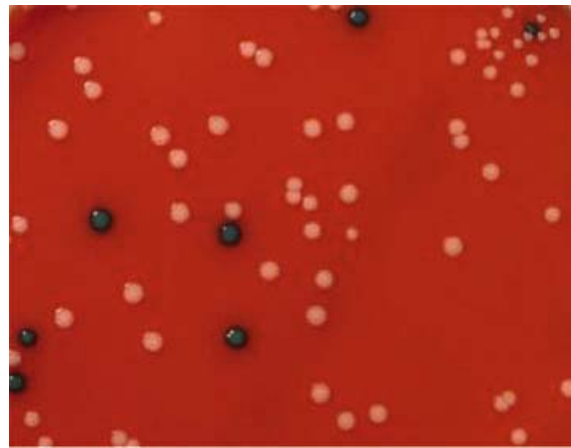
Fig. 1. *Listeria* spp. grown on RAPID'L. MONO medium. *Listeria monocytogenes* appears as blue colonies while other *Listeria* spp. appear as white colonies. Reproduced with permission from BIO-RAD, Marnes La Coquette, France.
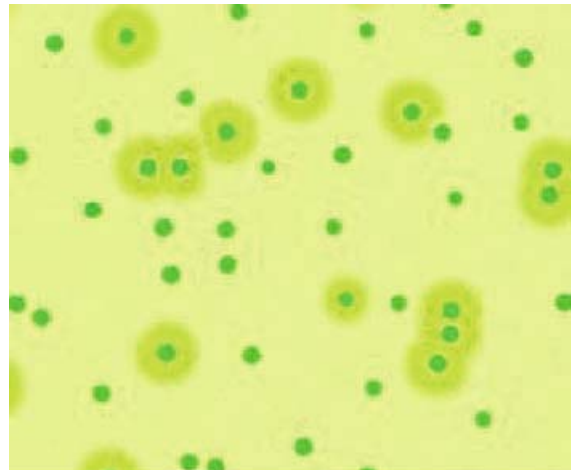
Fig. 2. *Listeria* spp. grown on ALOA (Agar *Listeria* Ottavani and Agosti) chromogenic medium. *Listeria monocytogenes* colonies are surrounded by a halo while other *Listeria* spp. are not. Reproduced with permission from AES Laboratoire, Ker Lann, France.

et al., 1995). Identification of *Listeria* spp. is based on a few biochemical markers and on hemolysis (Table 1). *Listeria* spp. can be identified using an API-*Listeria*® test, a set of 10 biochemical tests, including arylamidase, which discriminates pathogenic *L. monocytogenes* from nonpathogenic *L. innocua* (Swaminathan et al., 1995) (Fig. 3). Hemolysis is produced by *L. monocytogenes* but also by *L. ivanovii* and *L. seeligeri*. Hemolysis of *L. monocytogenes* is narrow and does not develop beyond the edge of colonies. It is due to listeriolysin O (LLO), a β-hemolysin known to be a major virulence factor. Hemolysis of *L. ivanovii* is wider, often bizonal, with a first ring of complete hemolysis and a

Table 1. Biochemical properties of *Listeria* species.

Property	*L. monocytogenes*	*L. ivanovii*	*L. seeligeri*	*L. innocua*	*L. welshimeri*	*L. grayi*
β-Hemolysis	+	+	+	−	−	−
CAMP reaction						
S. aureus	+	−	+	−	−	−
R. equi	−	+	−	−	−	−
Acid production from						
Mannitol	−	−	−	−	−	+
α-Methyl-D-mannoside	+	−	−	+	+	+
L-Rhamnose	+	−	−	V	V	−
Soluble starch	−	−	ND	−	ND	+
D-Xylose	−	+	+	−	+	−
Ribose	−	V	−	−	−	+
Hippurate hydrolysis	+	+	ND	+	ND	−
Reduction of nitrate	−	−	−	−	−	+

Symbols and abbreviations: +, present; −, absent; CAMP, Christie. Atkins, Munch-Peterson; V, variable; and ND, not determined.
From Swaminathan et al. (1995).

Fig. 3. The API-*Listeria*® test. Discrimination between *L. monocytogenes* (upper panel) and *L. innocua* (lower panel). From Allerberger et al. (2003), with permission.

second ring of incomplete hemolysis. *Listeria seeligeri* is weakly hemolytic (Fig. 4). Hemolysis of *L. ivanovii* and *L. seeligeri* is due to listeriolysin homologues, ivanolysin O and seeligerolysin O, respectively, and for *L. ivanovii* to the sphingomyelinase SmcL (Leimeister-Wachter and Chakraborty, 1989; Vazquez-Boland et al., 1989; Haas et al., 1992; Gonzalez-Zorn et al., 1999). The natural hemolysis of Listeriae is specifically enhanced by the products released by other hemolytic bacteria, *Staphylococcus aureus* for *L. monocytogenes* and *L. seeligeri*, or *Rhodococcus equi* for *L. ivanovii* in the CAMP test (Christie, Atkins, Munch-Petersen). In this assay, the streaking of Listeriae and *S. aureus* or *Rhodococcus equi* in close proximity and perpendicularly gives rise to a characteristic shovel-shaped patch of synergistic hemolysis (Swaminathan et al., 1995). In *L. ivanovii*, the bizonal hemolysis and the effect observed in the CAMP test are due to the SmcL phospholipase (Gonzalez-Zorn et al., 1999) (Fig. 5).

Other approaches, such as growth on chromogenic selective media, Rapid L-mono, or ALOA, allow identification of *L. monocytogenes*. Identification on Rapid L-mono plates is based on chromogenic detection of phosphatidylinositol (PI) phospholipase C (PLC-A) and on xylose fermentation differentially produced by *Listeria* species. *Listeria monocytogenes* appears as blue colonies (phospholipase C [PLC] positive) without halo (xylose negative), while *L. ivanovii* appears as blue colonies surrounded by a yellow halo (PLC and xylose positive). Other *Listeria* species appear as white colonies. In addition, chemiluminescent DNA probe assays allow rapid confirmation of *L. monocytogenes* from primary isolation colonies. Finally, sequencing of the 16S rRNA, ribotyping, or pulsed-field gel electrophoresis (PFGE), can be used as an identification method for species determination or subdivision within species (Brosch et al., 1996; Allerberger, 2003).

Typing

Although different methods are available to characterize isolates *L. monocytogenes* [phage typing,

ribotyping, DNA macro-restriction analysis, random amplified polymorphic DNA (RAPD) and multi-locus enzyme electrophoresis (MEE)], serotyping and DNA macro-restriction analysis are methods of choice for outbreak investigations. The 16 serovars of *Listeria* are based on the expression of somatic (O) and flagellar (H) antigens (Table 2), with 13 *L. monocytogenes* serovars that can cause disease. Approximately 95% of the human isolates belong to serovars 4b, 1/2a, and 1/2b. Strikingly, serovars 4b are found in most of the invasive foodborne outbreaks worldwide and in up to 50% of the sporadic cases of listeriosis, while serogroups 1/2 (including 1/2a, 1/2b and 1/2c) are mostly associated with sporadic cases (Rocourt and Cossart, 1997). More recently, multiplex PCR was proposed as an alternative to serotyping (Doumith et al., 2004b). The method allows to differentiate the major serovars, 1/2a, 1/2b, 1/2c and 4b, of *L. monocytogenes*.

Techniques based on phenotypic differentiation (phage typing and bacteriocinotyping) or on molecular characterization (MEE and DNA typing methods) were developed to further characterize strains of the same serovar. Phage typing is based on the testing of the isolates for their sensitivity to a set of phages isolated from lysogenic *Listeria* strains. MEE allows the determination of a pattern of enzyme activities that are specific for *Listeria* subtypes. Both techniques have been successfully applied for epidemiological studies. Molecular methods, such as characterization of chromosomal DNA by restriction endonuclease analysis, ribotyping, RAPD, or PFGE are also used to type *Listeria* strains. PFGE is the most discriminatory method to identify *L. monocytogenes* and is recommended, together
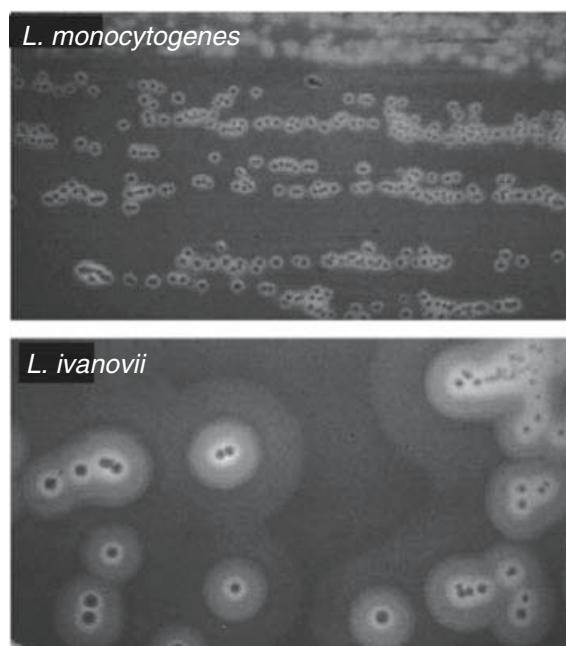
Fig. 4. Hemolysis on blood agar plates after 24 h. *Listeria monocytogenes* produces a narrow zone of hemolysis and *L. ivanovii* produced a wider bizonal hemolysis. From Vazquez-Boland et al. (2001), with permission.
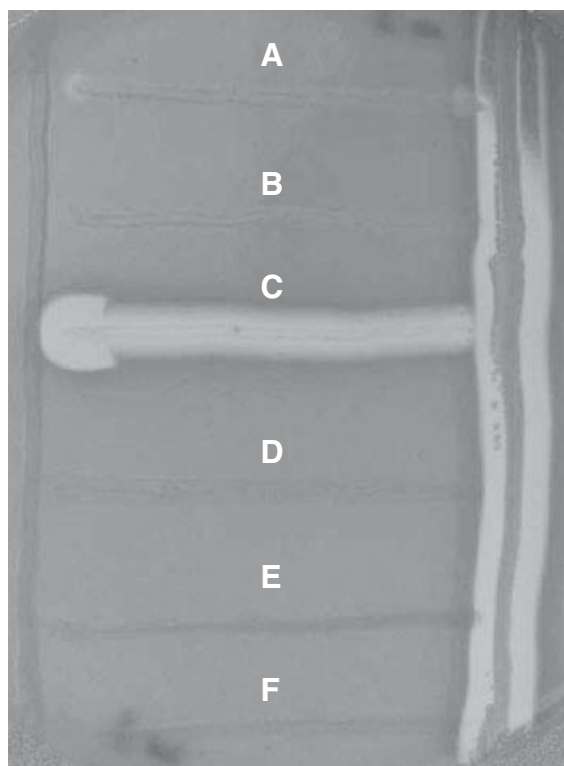
Fig. 5. The CAMP (Christie, Atkins, Munch-Peterson) test. Blood agar plates streaked horizontally with *L. monocytogenes* (A), *L. seeligeri* (B), *L. ivanovii* (C), *L. innocua* (D), *L. grayi* (E) and *L. welshimeri* (F) or vertically with *Rhodococcus equi* (left) or *Staphylococcus aureus* (right). Adapted from Allerberger et al. (2003), with permission.

Table 2. Serovars of the genus *Listeria*.

L. monocytogenes	*L. ivanovii*	*L. seeligeri*	*L. innocua*	*L. welshimeri*	*L. grayi*
1/2a, 1/2b, and 1/2c	5	1/2a, 1/2b, and 1/2c		1/2b	
3a, 3b, and 3c					
4a, 4ab, 4b, 4c, 4d, and 4e		4b and 4d	4ab	4c	
		6b	6a and 6b	6a and 6b	
7		US*	US	US	S

Abbreviations: US, undesignated serovar; S, specific; and * is both US and S.
From Swaminathan et al. (1995).

with serotyping, for epidemiological surveillance (Swaminathan et al., 1995; Brosch et al., 1996).

Serodiagnosis and Determination of Pathogenicity

Serologic tests cannot be used directly for diagnosis because of an antigenic cross-reactivity between *L. monocytogenes* and other Gram-positive bacteria (Swaminathan et al., 1995). However, detection of anti-listeriolysin antibodies, after adsorption on streptolysin O of *Streptococcus pyogenes*, is a good indicator of infection (Berche et al., 1990). Determination of the pathogenicity of Listeriae in animal models is not used routinely for identification (Swaminathan et al., 1995).

Physiology

Growth Conditions: pH, Temperature and Salts

Listeriae are facultative anaerobic bacteria, a property that might be advantageous during infection. They grow perfectly well in laboratory aerobic conditions. Although BHI (brain heart infusion) is the medium of choice for growth in vitro, Listeriae can grow in several other media such as Luria broth (LB). A minimal medium previously used (Premaratne et al., 1991) was recently readapted based on the analysis of the genome sequence of *L. monocytogenes* (Tsai and Hodgson, 2003). One of the key properties of *L. monocytogenes* resides in its ability to grow in a wide range of temperatures (1–45°C), which favors resistance and enrichment in foods. However, optimal growth occurs at 30–37°C. Listeriae are killed at 60°C, making pasteurization a good technique to eliminate the bacteria from dairy products (Seeliger and Jones, 1986). Analysis of *L. monocytogenes* grown at different temperatures and of cold-sensitive mutants revealed that temperature adaptation involves modifications of the fatty acid composition of the bacterial membrane (Annous et al., 1997). Listeriae also resist relatively extreme pH and salt conditions (pH 4.5–9 and 10% NaCl), but optimal growth occurs at neutral pH and 0.5% NaCl (McClure et al., 1991). The striking resistance of Listeriae to harsh external conditions accounts for its wide distribution in multiple habitats and explains the increasing numbers of food-borne outbreaks in industrialized countries.

Nutrients

Carbohydrates, amino acids (cysteine, glutamine, isoleucine, leucine and valine) and vitamins (biotin, riboflavin, thiamine and thioctic acid) are required for *Listeria* growth (Seeliger and Jones, 1986). An extensive study of the behavior of several auxotrophic mutants revealed that threonine, adenine or phenylalanine–tryptophan-tyrosine auxotrophs were less virulent (Marquis et al., 1993). Iron and some amino acids (arginine, histidine, methionine and tryptophan) stimulate *L. monocytogenes* growth. Activated charcoal or cellobiose have no effect on growth but modulate the expression of virulence genes (Cowart and Fosters, 1985; Seeliger and Jones, 1986; Park and Kroll, 1993; Ripio et al., 1996; Huillet et al., 1999).

Inhibitory Agents and Antibiotics

Virulent *Listeria* strains are resistant to bile (up to 40%) on agar plates. This is partly due to the expression of a bile salt hydrolase, encoded by the *bsh* gene (Dussurget et al., 2002) and other bile tolerance genes encoded by the locus *btlA* (Begley et al., 2003). Disinfectants are active on Listeriae allowing efficient treatment of surfaces in contact with food, but serum and milk reduce their bactericidal activities, emphasizing the attention drawn to hygiene in food industries (Best et al., 1990).

While antibiotic sensitivity might vary between strains, most Listeriae are sensitive to many antibiotics, such as aminoglycosides, tetracyclines, macrolides, chloramphenicol and penicillins. Some of them are used to treat listeriosis, e.g., usually ampicillin in combination with an aminoglycoside (Charpentier et al., 1995; Hof et al., 1997; Troxler et al., 2000). *Listeria* strains are naturally resistant to antibiotics such as cephalosporins or sulfonamides. Resistance to nalidixic acid is exploited to enrich samples in *L. monocytogenes*, and most enrichment media contain this antibiotic. Although antibiotic resistance of Listeriae is not yet a concern for clinics, new tetracycline- or trimethoprim-resistant strains have recently emerged (Poyart-Salmeron et al., 1990; Poyart-Salmeron et al., 1992; Charpentier et al., 1995; Charpentier and Courvalin, 1999).

The Human Disease: Epidemiological and Clinical Aspects

Epidemiology

Listeria monocytogenes had been isolated in humans as early as the 1920s (Murray et al., 1926; Pirie, 1927). Nevertheless, it was not identified as an important cause of neonatal infection until after World War II in Germany (Potel, 1952). The

development and use of immunosuppressive agents in the second half of the twentieth century led to the identification of listeriosis cases in immunocompromized patients (Louria et al., 1967). Subsequently, the development of highly immunosuppressive therapies for organ or bone marrow transplantations led to a great expansion of the immunocompromized population and the subsequent identification of immunosuppression as a major risk factor for listeriosis (Stamm et al., 1982). More recently, the human immunodeficiency virus (HIV) epidemic has markedly enlarged the immunodeficient population, with a relative risk of developing listeriosis 500 times higher in acquired immunodeficiency syndrome (AIDS) patients as compared to the general population (Jurado et al., 1993). Globally, human listeriosis remains a rare disease, and its prevalence is declining in industrialized countries in which food control measures have been implemented. The attack rate is considered to be around 7 per million, leading to 2500 cases and around 500 deaths per year in the United States (Gellin et al., 1991; Lorber, 1997; Vazquez-Boland et al., 2001; Wing and Gregory, 2002). Infection is more common in children (100 cases per million) and elderly (14 cases per million). Pregnant women are 20 times more likely to develop listeriosis than the general population (Broome, 1993).

It has been observed very early in ruminants that oral ingestion of *L. monocytogenes* was associated with listeriosis (Low and Renton, 1985). This led to the hypothesis that *L. monocytogenes* was also a human foodborne pathogen. However, not until the 1980s was the validity of this hypothesis formally demonstrated, and it has now been widely confirmed by other investigators (Schlech et al., 1983). A number of listeriosis outbreaks and sporadic cases consequent to the ingestion of contaminated food products have been reported, as well as the origin of contamination (Table 3). Indeed, vegetables can become contaminated from the soil or from manure used as fertilizer, and animals can carry the bacterium asymptomatically and contaminate foods of animal origin, such as meats and dairy products. *Listeria monocytogenes* is found in a variety of raw foods, such as uncooked meats and vegetables, or processed foods that become contaminated after processing, such as soft cheeses and cold cuts at deli counters (Fleming et al., 1985; Linnan et al., 1988; Schwartz et al., 1989; Riedo et al., 1994; Bula et al., 1995; Salamina et al., 1996; Dalton et al., 1997; Goulet et al., 1998; Aureli et al., 2000; Ooi et al., 2005). Unpasteurized milk or foods made from unpasteurized milk may contain the bacterium. *Listeria monocytogenes* is killed by pasteurization and cooking. However, in certain ready-to-eat foods such as hot dogs and deli meats, contamination may occur after cooking before packaging. Government agencies and the food industry have taken steps to reduce contamination of food by *L. monocytogenes*. The United States Food and Drug Administration (FDA) (http://www.fda.gov/) and the United States Department of Agriculture (USDA) (http://www.usda.gov) monitor food regularly. When a processed food is found to be contaminated, food monitoring and plant inspection are intensified, and if necessary, the implicated food is recalled (for details, see the web sites of the World Health Organization (http://www.who.int/foodsafety/en/), Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov), (http://www.foodsafety.gov/~fsg/fsgprobs.html{Gateway to Government Food Safety Information} or http://www.afssa. fr{Agence française de sécurité sanitaire des aliments}).

Most human foodborne infections are associated with high prevalence but low morbimortality. The situation is different for human listeriosis, which is a rare but very serious infec-

Table 3. Main listeriosis outbreaks in Europe and the United States.

Year	Location	Number of cases	Perinatal cases (%)	Mortality rate (%)	Source of contamination
1980–1981	Canada	41	83	34	Cole slaw
1983	New England, USA	49	14	29	Pasteurized milk
1983–1984	Switzerland	57	9	32	Soft cheese
1985	California, USA	142	65	34	Mexican cheese
1986–1987	Pennsylvania, USA	36	11	44	Unknown
1989	Connecticut, USA	10	20	10	Shrimps
1992	France	38	82	32	Deli meat (rillettes)[a]
1993	Italy	39	Unknown	Unknown	Rice salad
1994	Illinois, USA	45	Unknown	Unknown	Chocolate milk
1997	Italy	1566	Unknown	Unknown	Corn salad
1998–1999	United States	1001	Unknown	Unknown	Hot dogs
1999	France	32	12	21	Pork deli meat
2002	Illinois, USA	43	28	31	Turkey deli meat

[a]Rilettes, a spread similar to paté, usually made of pork.

tion, associated with a mortality of up to 30%, even when an adequate treatment is administered. This accounts for the high economic impact associated with listeriosis despite its relative low prevalence (Table 4). Moreover, listeriosis remains under-diagnosed, particularly at its early stages, and this leads to delay in the administration of antimicrobial therapy, which is absolutely critical for a favorable outcome, contrary to most other foodborne infections (Lorber, 1997; Wing and Gregory, 2002). More detailed informations concerning epidemiology can be found at http://www.cdc.gov/ncidod/dbmd/ diseaseinfo/listeriosis_t.htm; http://www.who.int/ foodsafety/micro/jemra/assessment/listeria/en/; http://www.invs.sante.fr/beh/2004/09/index.htm.

Clinical Manifestations

Listeria monocytogenes infects humans through the ingestion of contaminated food products. The bacteria can cross the intestinal barrier and disseminate from the mesenteric lymph nodes to the spleen and the liver, from which they may reach the brain or the placenta, resulting in meningitis or encephalitis in immunocompromized patients, abortions in pregnant women, or generalized infections in infected neonates (*granulomatosis infantiseptica*) (Fig. 6). If not controlled properly by the immune system, *L. monocytoge-*

nes infection may also cause septicemia. Highly contaminated food products also infect healthy individuals, resulting mainly in gastroenteritis (Aureli et al., 2000; Vazquez-Boland et al., 2001; Wing and Gregory, 2002; Ooi et al., 2005).

DIGESTIVE MANIFESTATIONS Studies reported by the CDC have shown that 11% of the food samples collected in the framework of food monitoring programs were contaminated with *L. monocytogenes* and that *L. monocytogenes* grew from at least one refrigerator sample of 64% of patients with listeriosis (Pinner et al., 1992). For long, this first step of the infectious process—consumption of *L. monocytogenes*-contaminated food—was considered to be almost always clinically silent. However, it was clearly demonstrated that it may lead to the development of a genuine gastroenteritis (with digestive signs such as nausea, aqueous or bloody diarrhea, abdominal pain, and fever) with an attack rate of up to 70%, particularly in cases of high inocula (Dalton et al., 1997; Schlech, 1997; Aureli et al., 2000). Although a number of *L. monocytogenes* associated gastroenteritis outbreaks have been reported, cases of sporadic enteritis appear to be relatively rare (Schlech et al., 2005). The importance of the inoculum size in the onset of these early clinical signs is supported by the results of oral

Table 4. Cost of foodborne infections in the United States in 1992.

Bacteria	Number of cases in the United States in 1992	Global cost ($US)	Cost per patient ($US)
Salmonella	1,920,000	1,388,000,000	723
Campylobacter jejuni	2,100,000	961,500,000	458
Escherichia coli O157H7	14,058	388,000,000	27,600
Listeria monocytogenes	1,550	221,000,000	142,581

For detailed information about the economics of foodborne diseases, see USDA listing of foodborne diseases.
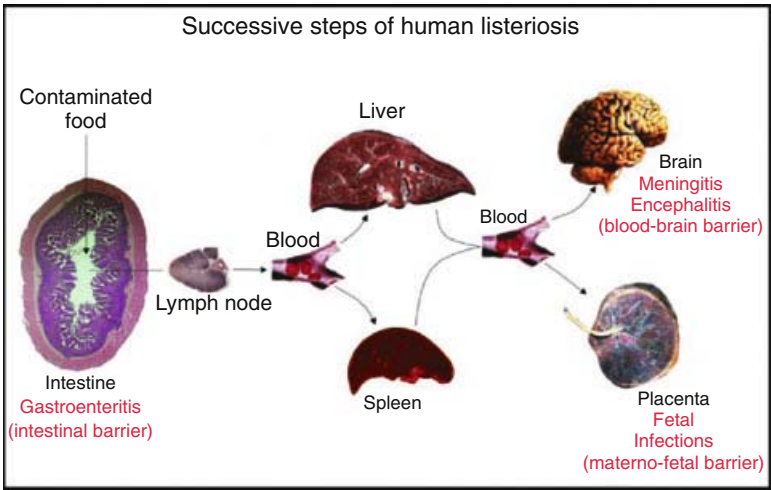
Fig. 6. Successive steps of listeriosis. Organs affected by the infection, symptoms and epithelial barriers crossed by *L. monocytogenes*.

challenges in healthy nonhuman primates, in whom the ingested inoculum had to be higher than 109 organisms to produce a detectable clinical effect (Farber et al., 1991b). Whether these digestive clinical signs mostly account for a toxic effect of *L. monocytogenes* on the intestinal tissue or for the crossing and invasion of the mucosal barrier by *L. monocytogenes* is not known. These digestive manifestations are usually self-limited and resolve spontaneously.

BACTEREMIA In the population at risk for invasive listeriosis, signs accounting for generalized infection most frequently occur after an incubation period that can be very long (10–70 days). Hematogenous seeding of the pathogen resulting in *L. monocytogenes* bacteremia might be responsible for an influenza-like febrile illness, accompanied by myalgias, arthralgias, headache and backache but might also be clinically silent (Goulet and Marchetti, 1996).

PREGNANCY-ASSOCIATED LISTERIOSIS During pregnancy, particularly the third trimester when pregnancy-associated immunosuppression is the most intense, benign common cold symptoms may be due to *L. monocytogenes*-associated bacteremia and should be treated *a priori* as listeriosis because of the seriousness of possible obstetrical and neonatalogical complications. The specific complications of listeriosis during pregnancy lie in the ability of *L. monocytogenes* to cross the maternofetal barrier and result in placental abscesses, chorioamnionitis, and finally infection of the fetus. This infection is responsible for premature labor, stillbirth, abortion, and neonatal infection, with high mortality. Disseminated infection to the fetus is called granulomatosis infantiseptica and is characterized by the widespread presence of microabscesses and granulomas in liver, spleen and skin. The highest concentrations of *L. monocytogenes* being encountered in the gut and in the lung, infection might be amplified through ingestion of contaminated amniotic fluid rather than solely as a consequence of the hematogenous transplacental route (Lorber, 1997; Schlech, 2000). In cases of neonatal contamination during parturition, a primary septicemic syndrome similar to that associated with *Streptococcus agalactiae* infection occurs sometimes with purulent conjunctivitis and disseminated papular rash evocative of neonatal listeriosis and late-onset meningitis. *Listeria monocytogenes* meningitis is one of the three major causes of meningitis in neonates (Lorber, 1997; Dawson et al., 1999; Schlech, 2000; Lecuit and Cossart, 2001a).

INFECTION OF THE CENTRAL NERVOUS SYSTEM *Listeria monocytogenes* has, in addition to its ability to cross initially the intestinal barrier and the maternofetal barrier in pregnant women, the capacity to cross the blood-brain barrier and reach the central nervous system (CNS). Interestingly, it has been proposed, based on animal studies, that infected bone marrow myeloid cells may promote *Listeria* invasion of the CNS (Join-Lambert et al., 2005). *L. monocytogenes* capacity to cause both acute meningitis and parenchymal brain infection differentiates it from other bacteria frequently responsible for meningitis such as *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae*. Among all bacterial meningitides, *L. monocytogenes* meningitis has the highest mortality rate (22%). *L. monocytogenes* meningitis accounts for 11% of all bacterial meningitis and was the second most common cause of meningitis after *Streptococcus pneumoniae* meningitis in patients older than 50 years (Lecuit and Cossart, 2001a). The clinical features of *L. monocytogenes* meningitis differ from those of other bacterial meningitides in that *L. monocytogenes* meningitis may have a subacute course, and be associated with abnormal movements, seizures, and alteration of consciousness. The onset of such signs is evocative of dissemination to the brain parenchyma. Two different types of dissemination to the brain can occur: encephalitis or intraparenchymal brain abscesses. Encephalitis usually involves particularly the rhombencephalon and clinically appears as a meningitic syndrome that is complicated with cranial nerve deficits, a cerebellar syndrome, or central motor and sensitivity deficits. In two-thirds of the cases, blood cultures are positive. Cerebrospinal fluid culture is positive in half of the cases. The onset of rhombencephalitis is not confined to the immunocompromised host and may also be reported in otherwise healthy adults (Armstrong and Fung, 1993). In a small proportion of *L. monocytogenes* CNS infections (10% with half of them in immunocompromized patients), macroscopic brain abscesses are observed. *Listeria monocytogenes* brain abscesses are preferentially located in subcortical areas, thalamus, pons or medulla. These unusual locations are evocative of their listerial origin (Lorber, 1997; Schlech, 2000). Both encephalitis and parenchymal abscesses are associated with high neurological sequelae and mortality rates.

LOCALIZED INFECTIONS Rare cases of *L. monocytogenes* endocarditis have been reported, accounting for less than 10% of all *L. monocytogenes* infections and occurring in patients at risk for endocarditis. Rare localized infections in patients at risk for listeriosis have also been reported and include infections due to direct inoculation (conjunctivitis, skin infection or

lymphadenitis), digestive involvement (peritonitis or cholecystis), or hematogenous seeding (abscesses of liver and spleen, pleuropulmonary infections, joint infection and osteomyelitis, pericarditis, myocarditis, arteritis or endophtalmitis) (Lorber, 1997; Schlech, 2000; Lecuit and Cossart, 2001a).

Genetics of *Listeria*

Genetic Elements of *Listeria*

PHAGES Lysogenic bacteriophages have been isolated from *Listeria* species. They belong to the Siphoviridae and Myroviridae families and contain double-stranded DNA of about 40 kb. They do not appear to be involved in virulence, but some have been used to classify *Listeria* strains by phage typing (Cossart and Mengaud, 1989a; McLauchlin et al., 1996). Phages of *Listeria monocytogenes* are, with a few exceptions, specific with respect to the serogroup of the host cells. The major serogroups (1/2 and 4) can be differentiated on the basis of somatic antigen composition and sugar substitution of cell wall teichoic acids (TAs) (Fiedler and Ruhland, 1987). These carbohydrates also serve as a primary receptor for the serogroup specific phages (Wendlinger et al., 1996). In 2000, the complete nucleotide sequence and structural analysis of the serogroup 1/2-specific bacteriophage A118 were reported (Loessner et al., 2000). The A118 genome is a 40.8-kb, linear, circularly permuted, terminally redundant collection of double-stranded DNA molecules. Site-specific integration of the A118 prophage occurs into a gene homologous to *comK* from *Bacillus subtilis*, resulting probably in the inactivation of this gene. Comparative analysis of the A118 genome structure with other bacteriophages revealed local, but sometimes extensive similarities with a number of phages spanning a broader phylogenetic range of low G+C bacteria, implying relatively recent exchange of genes and genetic modules (Loessner et al., 2000). The genome and the proteome of a serogroup-4-specific phage, PSA, were described in 2003 (Zimmer et al., 2003). PSA is a temperate phage isolated from *L. monocytogenes* strain Scott A, a clinical epidemic strain. The genome of the bacteriophage PSA is 37.6 kb long and contains 57 open-reading frames, which are organized into three major transcriptional units. The PSA integration site is *attB* located at the 3′ end of the single-copy tRNA^{Arg} gene, where phage nucleotides reconstitute its function (Lauer et al., 2002). Bioinformatics revealed only few similarities of PSA with the *Listeria* phage A118. The analyses also revealed the first case of a +1 frameshifting among dsDNA phages, and the utilization of a 3′ pseudoknot to stimulate such an event (Zimmer et al., 2003). On the basis of the analyses of these two bacteriophages and the identification of their respective integration sites, two site-specific integration vectors, pPL1 and pPL2, which utilize the listeriophage U153 integrase, were constructed (Lauer et al., 2002) (Fig. 7). pPL1 integrates in the *comK* attachment site and pPL2 uses the PSA attachment site in the tRNA^{Arg} gene. These plasmids were used in *L. monocytogenes* enabling complementation experiments (Lauer et al., 2002).

PLASMIDS Many strains of *Listeria* species contain one large cryptic plasmid. Several plasmids of various sizes were detected in *Listeria*, but hybridization and restriction analysis revealed a high degree of homology between them (Kolstad et al., 1991). In certain cases, *L. monocytogenes* plasmids were associated with cadmium resistance (Lebrun et al., 1992) or antibiotic resistance (Charpentier and Courvalin, 1999). Studies on the conjugative abilities of the plasmids associated with antibiotic resistance showed that they were transferable to *Listeria* spp. as well as to other Gram-positive bacteria and suggested that they were probably acquired from the *Enterococci-Streptococci* group (Poyart-Salmeron et al., 1990; Lebrun et al.,
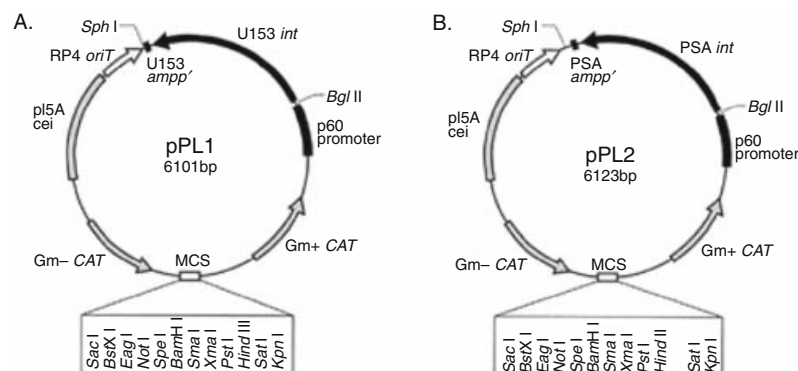
Fig. 7. Maps of the site-specific integration vectors pPL1 and pPL2. Chloramphenicol resistance genes (CAT) and origin of replication in *E. coli* are represented in gray; RP4 origin of transfer is represented in white. The U153 and PSA integrases (U153*int* and PSA*int*) and the p60 promoter of *L. monocytogenes* are represented in black. MCS is the multicloning site. Adapted from Lauer et al. (2002), with permission.

1992; Charpentier and Courvalin, 1999). Plasmid vectors originating from *Bacillus subtilis* or *Escherichia coli* replicate in *Listeria* and thus are used for genetic studies in *L. monocytogenes*, including allelic exchange of chromosomal DNA, cloning, gene expression, or reporter gene fusion (Freitag, 2000).

Transposons Some *L. monocytogenes* strains harbor a natural transposon Tn*5422*, which contains 40-bp inverted repeats, two genes conferring cadmium resistance, and two genes encoding a transposase and a resolvase (Lebrun et al., 1994). *Listeria* can also naturally acquire *Enterococci-Streptococci* transposons from the Tn*1545*-Tn*916* family, conferring on strains tetracycline resistance and other resistance genes (Poyart-Salmeron et al., 1989; Poyart-Salmeron et al., 1992). The conjugative properties of these transposons were widely used for mutagenesis and constituted the first genetic tools to study *L. monocytogenes* virulence and physiology (Gaillard et al., 1986; Kathariou et al., 1987; Portnoy et al., 1988; Cossart et al., 1989b; Marquis et al., 1993). Contrary to conjugative transposons, nonconjugative transposons such as Tn*917* or Tn*917-lac* do not generate multiple insertions and are consequently more powerful tools (Cossart et al., 1989b). More recently, Tn*1545* or Tn*917*–derived tagged transposons were also used for signature-tagged mutagenesis in *L. monocytogenes*, allowing the identification of new virulence genes (Autret et al., 2001; Dramsi et al., 2004; Mandin et al., 2004).

Genetic Techniques and Tools

Genetic techniques used to modify the *Listeria* genome are derived from those (including conjugation of self-conjugative transposons or plasmids, and transformation of plasmids) utilized to modify other Gram-positive bacteria (Cossart and Mengaud, 1989a). Interestingly, *L. monocytogenes* itself has been used as a genetic vehicle to deliver functional genes into eukaryotic cells (Mollenkopf et al., 2001; Grillot-Courvalin et al., 2002; Pilgrim et al., 2003b).

Plasmid Vectors Several shuttle plasmid vectors from *Bacillus subtilis* have been used in *L. monocytogenes*. They contain multicloning sites and genes encoding antibiotic resistance, allowing genetic studies (Freitag, 2000).

Plasmids Preferentially Used for Complementation or Gene Expression Among the broad host range shuttle plasmids replicating in *Escherichia coli* and *L. monocytogenes*, pMK4 and pAM401 are the most extensively used for complementation of mutants obtained by transposon insertion or for expression of gene products via their own promoters (Sullivan et al., 1984; Wirth et al., 1986; Cossart et al., 1989b; Freitag, 2000) (Fig. 8). Similarly, derivatives of the conjugative plasmid, pAT18 (Trieu-Cuot et al., 1991), allowed complementation of deletion mutants (Gaillard et al., 1991; Dramsi et al., 1995) as well as green fluorescent protein (GFP) expression (Fortineau et al., 2000). pBR474 derivatives have also been used to successfully express *L. monocytogenes* proteins. They combine the advantage of an easy selection of recombinants (because of the high natural sensitivity of *Listeria* strains to chloramphenicol) and a high level expression of inserts (because of the presence of a strong promoter) (Lecuit et al., 1997). The recent construction of a plasmid, pLIV1, carrying an isopropyl-β-D-thiogalactoside (IPTG)-inducible SPAC pro-
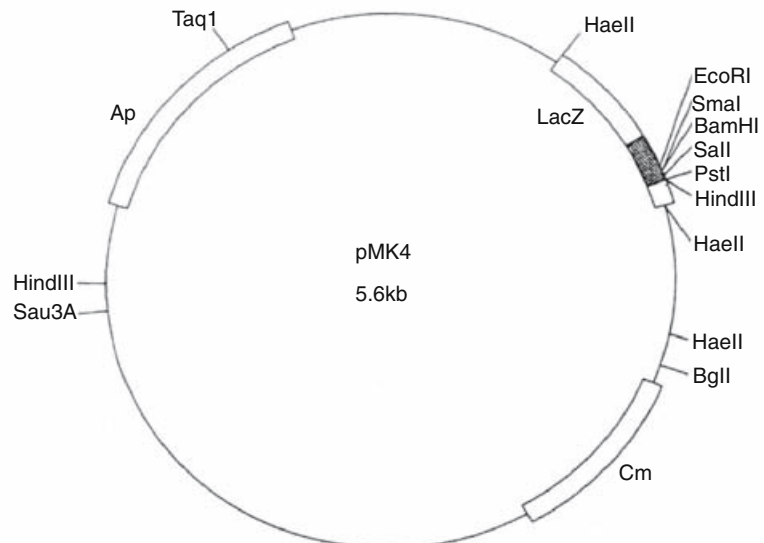
Fig. 8. Physical map of the pMK4 plasmid. The ampicillin (Ap) and chloramphenicol (Cm) resistance genes allow selection in *E. coli* and *L. monocytogenes*, respectively. From Sullivan et al. (1984), with permission.

moter permitted in vivo studies in which expression of *L. monocytogenes* genes were followed intracellularly (Freitag and Jacobs, 1999; Dancz et al., 2002). The construction of integrative phage-derived plasmids, pPL1 and pPL2, has also allowed complementation of deletion mutants (Fig. 7). Their advantage resides in their ability to integrate at a specific location, which may facilitate fine genetic analysis of mutants or gene transfer in *L. monocytogenes* (Lauer et al., 2002).

Plasmids Preferentially Used for Allelic Exchange Thermosensitive vectors such as pKSV7 (Smith and Youngman, 1992) (Fig. 9) or pAUL-A (Schaferkordt and Chakraborty, 1995) allow allelic exchange, in frame deletion mutagenesis (Brundage et al., 1993; Dramsi et al., 1995; Domann et al., 1997), or site-specific mutagenesis (Boujemaa-Paterski et al., 2001). A novel thermosensitive plasmid, pMAD, harboring the β-galactosidase gene fused to the strong constitutive promoter of *clpB* from *Staphylococcus aureus* (Fig. 10), facilitates the screening and the generation of allelic exchanges (Arnaud et al., 2004).

Transposons Different transposons were used for random mutagenesis in *L. monocytogenes*. Conjugative transposons from the Tn*1545*-Tn*916* family or Tn*917* transposons were initially exploited to generate libraries of mutants, which allowed the characterization of several virulence factors of *L. monocytogenes* (Gaillard et al., 1986; Kathariou et al., 1987; Portnoy et al., 1988;

Cossart et al., 1989b). More recently, tagged Tn*1545* derivatives carried by an integrative vector were utilized for signature-tagged mutagenesis in *L. monocytogenes*, allowing the identification of new virulence genes (Autret et al., 2001). The use of Tn*917* or Tn*917* derivatives (pTV1, pTV32, pTLV1 and pTLV3), carried on vectors with temperature-sensitive origins, facilitated the screening and the generation of large-scale libraries of mutants, in particular those of *L. monocytogenes* virulence genes (Camilli et al.,
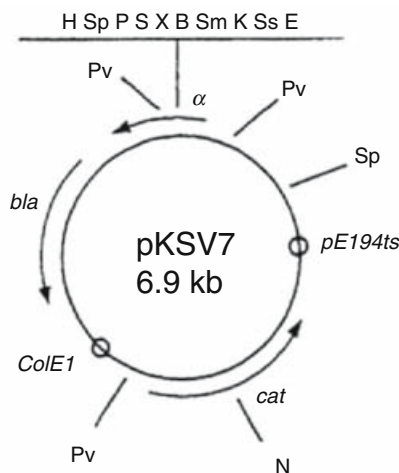
Fig. 9. Map of the pKSV7 thermosensitive plasmid. *bla* is the β-lactamase gene of pUC18; *cat* is the chloramphenicol acetylase transferase gene of pC194; α is the *lac* alpha-complementing gene fragment of pUC18. From Smith and Youngman (1992), with permission.
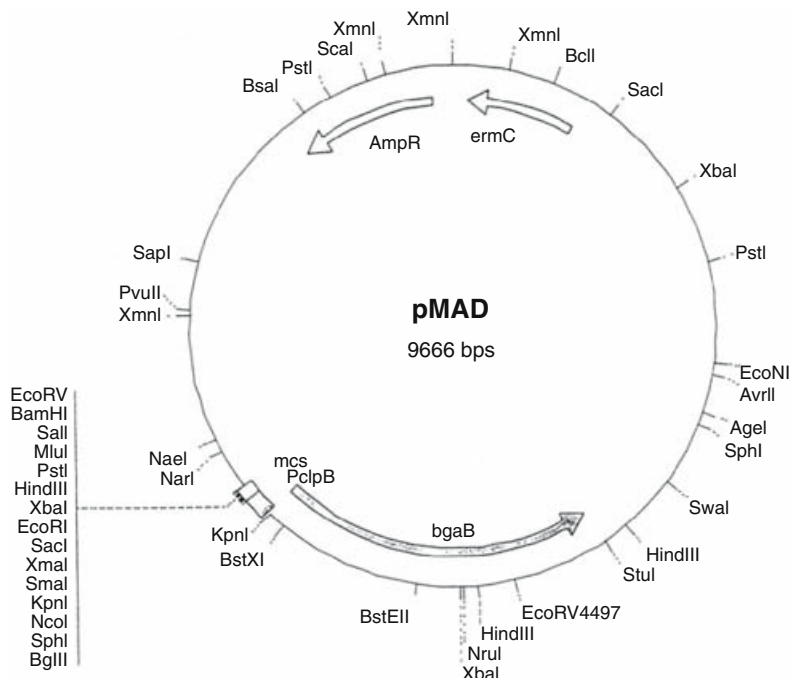
Fig. 10. Map of the pMAD plasmid. The ampicillin (*ampR*) and erythromycin (*ermC*) resistance genes allow selection in *E. coli* and *L. monocytogenes*, respectively. *bgaB* is the β-galactosidase gene fused to the strong constitutive promoter of *clpB* from *Staphylococcus aureus* (PclpB). From Arnaud et al. (2004), with permission.

Fig. 11. Map of the pTV1, pTV31 and pTV32 plasmids. *erm* gene is the erythromycin resistance gene of the transposon Tn*917*; *cat* is the chloramphenicol acetylase transferase gene of pC194; *lacZ* is the promoterless *lacZ* gene of *E. coli*. From Perkins and Youngman (1986), with permission.
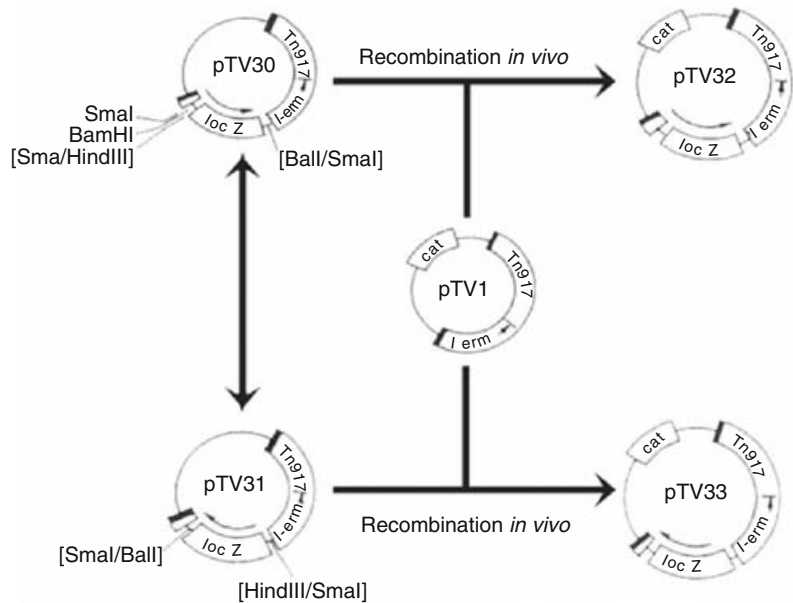
1990; Sun et al., 1990; Mengaud et al., 1991b) (Fig. 11). Some of these transposons have also the advantage of allowing the direct cloning of the flanking regions of the transposon insertion site (pTLV1 and pTLV3) or carry a promoterless *lacZ* gene permitting transcriptional gene fusion (pTV32) (Camilli et al., 1990; Francis and Thomas, 1997).

REPORTER GENES Transcriptional fusions to reporter genes, mostly *lacZ*, *lux* or GFP, have been used to monitor *Listeria* gene expression in vitro or intracellularly (Klarsfeld et al., 1994; Bubert et al., 1999; Freitag, 2000). More recently, a transposon carrying a modified version of the *lux* operon fused to promoterless *L. monocytogenes* genes (Francis et al., 2000) has opened a new way to monitor infection using in vivo bioluminescence imaging in mice (Hardy et al., 2002).

CONJUGATION Self-conjugative transposons, plasmids, and phage-derived vectors are used for genetic studies in *Listeria*. Most often, conjugation is performed on filters onto which donor and recipient bacteria are deposited (Cossart and Mengaud, 1989a). pAT18 vectors or derivatives were used for complementation studies of mutants affected in virulence determinants (Gaillard et al., 1991) or to generate *L. monocytogenes* expressing GFP, allowing their detection and tracking in vivo (Fortineau et al., 2000). Conjugative transposons of the Tn*1545*-Tn*916* family transfer from the donor bacteria to the recipient bacteria, where they integrate randomly into the chromosome. This strategy was the first used to

generate a large set of defective mutants (Gaillard et al., 1986; Kathariou et al., 1987; Portnoy et al., 1988; Cossart et al., 1989b; Marquis et al., 1993). Recently, conjugation of listeriophage-derived integrative vectors, pPL1 and pPL2, was successfully exploited for complementation studies of *L. monocytogenes* mutants (Lauer et al., 2002).

TRANSFORMATION Listeriae are not naturally competent, although competence genes have been found in *L. monocytogenes* genome (Glaser et al., 2001). However, transformation with plasmid DNA can be obtained on protoplasts or by electroporation (Vicente et al., 1987; Luchansky et al., 1988). Treatment of bacteria with low concentration of penicillin optimizes the efficiency of electroporation (Park and Stewart, 1990). Genetic studies resulting from *L. monocytogenes* transformation include allelic exchange of chromosomal DNA, complementation of defective genes, and reporter gene fusion (Cossart et al., 1989b; Dramsi et al., 1995; Dubail et al., 2000).

TRANSDUCTION Until recently, transduction was not possible in *L. monocytogenes*. It has now been successfully performed using bacteriophages that grow at 30°C but not at 37°C. Transduction at low multiplicity of infection, at 37°C, and in presence of citrate resulted in elimination of transductant and lysogeny, allowing the isolation of several *L. monocytogenes* transductants of different serotypes, and opening the way to new genetic approaches to modify the *Listeria* genome (Hodgson et al., 2000).
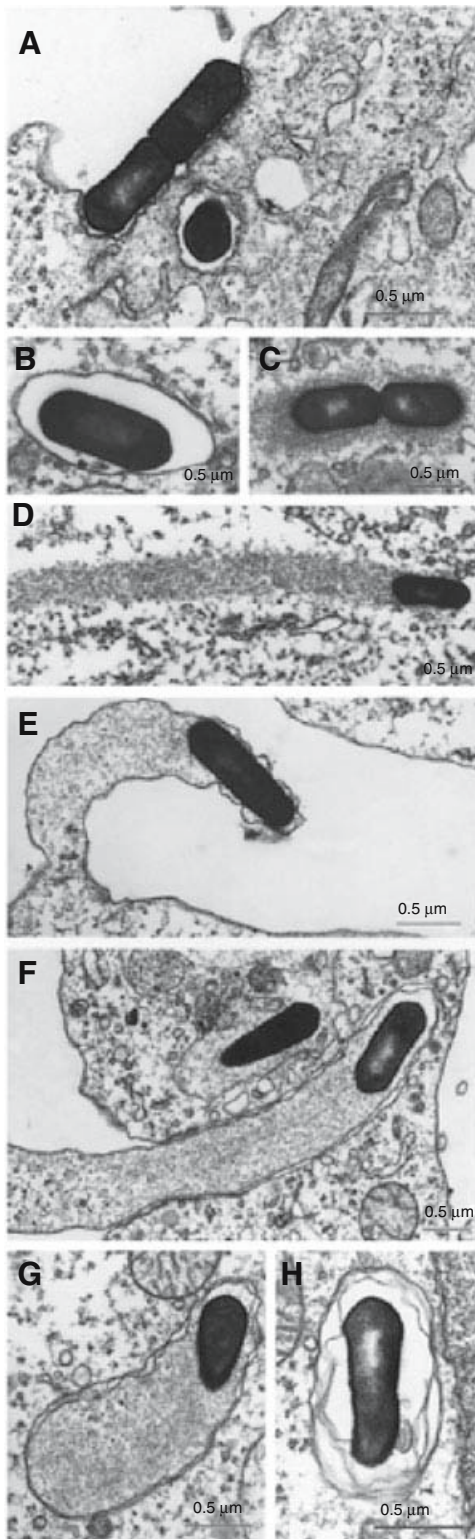
Fig. 12. Electron microscopy images of the successive steps of *L. monocytogenes* infection. Thin sections of cells infected with *L. monocytogenes* are examined by electron microscopy. A and B: entry and formation of the phagocytic cup; C and D: intracellular movements; E and F: cell-to-cell spread; G and H: formation and lysis of the two-membrane vacuole. From Cossart and Lecuit (1998), with permission.
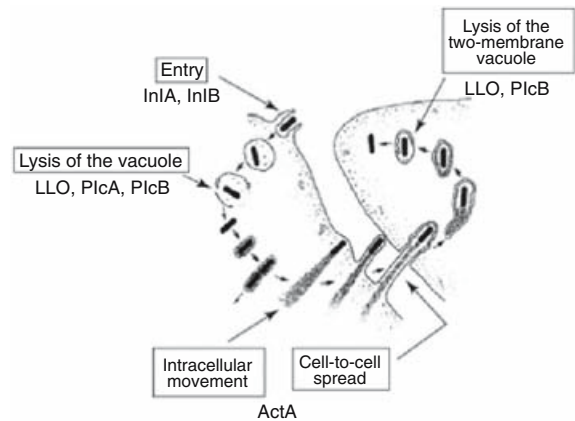
Fig. 13. Model for *L. monocytogenes* infection. The successive steps of *L. monocytogenes* infection as well as the bacterial factors are indicated. Adapted from Tilney and Portnoy (1989), with permission.

The Cell Biology of *L. monocytogenes* Infection

While our knowledge of the different steps leading to listeriosis remains rather elusive, the cell biology of the infectious process has been widely studied (Figs. 12 and 13). The main feature of *L. monocytogenes* is its capacity to enter into non-phagocytic cells and to survive and multiply in the cytosol of most cell types, including macrophages. Intracytoplasmic bacteria exploit the host cytoskeleton to move intracellularly through an actin-dependent process. (Lecuit and Cossart, 2001; Cossart and Sansonetti, 2004). Interestingly, it was shown that intracytosolic *L. monocytogenes* avoids recognition by the ubiquitin-dependent degradation system, as a possible consequence of its motility (Perrin et al., 2004). *L. monocytogenes* form protrusions that invade adjacent cells, allowing dissemination by direct cell-to-cell spread, without reaching the extracellular milieu, therefore avoiding the extracellular defenses, such as antibodies and complement (Lecuit and Cossart, 2001a; Cossart and Sansonetti, 2004). These features, including the avoidance of lysosomal enzymes and serum components, strongly account for the virulence of *L. monocytogenes*.

Bacterial Internalization

Both in vivo and in cultured cells, *L. monocytogenes* can be phagocytosed by macrophages (Harrington-Fowler et al., 1981; Campbell et al., 1994), neutrophils (Arnold and Konig, 1998), or dendritic cells (Guzman et al., 1995; Kolb-Maurer et al., 2000; Pron et al., 2001). *Listeria monocytogenes* can also induce its own internalization in a wide variety of nonphagocytic cells,

Fig. 14. Electron microscopy of *L. monocytogenes* invading Caco-2 cells. Scanning electron microscopy images of cells infected for 15 min (A) or 30 min (B) by *L. monocytogenes*. Transmission electron microscopy images of cells infected by *L. monocytogenes* at low (C) or high (D) magnification. Scale bar, 0.5 µm. From Mengaud et al. (1996), with permission.
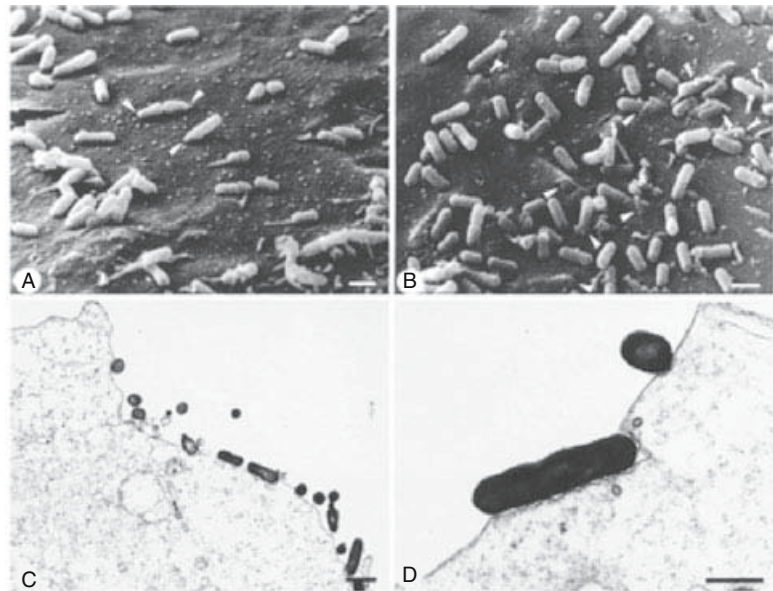
including enterocytes (Gaillard et al., 1987; Lecuit et al., 2001b), hepatocytes (Gaillard et al., 1996; Gregory et al., 1996b), fibroblasts (Portnoy et al., 1988), endothelial cells (Drevets et al., 1995; Greiffenberg et al., 1998; Parida et al., 1998) or glial cells of the CNS (Dramsi et al., 1998). Phagocytosis of *L. monocytogenes* in epithelial cells does not produce an extensive remodeling of the cell surface, as seen with *Salmonella-* or *Shigella*-induced ruffling (Swanson and Baer, 1996). Rather it occurs by a zipper-like mechanism, characterized by an intimate bacterium-cell membrane interaction, followed by the progressive invagination of the plasma membrane leading to the bacterial engulfment into the adjacent cell (Mengaud et al., 1996; Figs. 12A and 14). *Listeria* entry into non-phagocytic cells was shown to be dependent on membrane cholesterol (Seveau et al., 2004) and to exploit the clathrin-dependent endocytic cell machinery (Veiga and Cossart, 2005). The entry process is predominantly promoted by two leucine-rich repeat (LRR) proteins, InlA and InlB, expressed at the surface of *L. monocytogenes* and belonging to the internalin (Inl) multigenic family (Gaillard et al., 1991; Dramsi et al., 1995; Braun et al., 1999).

Intracellular Multiplication

After phagocytosis, the vacuole containing *L. monocytogenes* acidifies and, most often, is lysed by the bacterium in less than 30 min (Gaillard et al., 1987; Tilney and Portnoy, 1989; Fig. 12B). In macrophages, escape from the phagosome occurs for approximately 50% of the bacterial population and prevents bacterial destruction by the phagolysosomal components (Tilney and Portnoy, 1989; De Chastellier et al., 1994). It involves principally three bacterial proteins, the pore forming toxin, LLO (Gaillard et al., 1987), PLC-A (Camilli et al., 1993), and the phosphatidylcholine (PC) phospholipase C (PLC-B) (Grundling et al., 2003). Recently, it was suggested that lipoproteins might also participate in the bacterial escape from the phagosome (Reglier-Poupet et al., 2003a). In macrophages, *L. monocytogenes* delays phagosome maturation, which probably accounts for the vacuolar escape (Alvarez-Dominguez, 1997a). *L. monocytogenes* also inhibits the exchange activity of the endosomal trafficking regulator Rab5a, blocking the recruitment of lysosomal proteins to the phagosomes and avoiding the intraphagosomal killing of *L. monocytogenes* (Prada-Delgado et al., 2001; Prada-Delgado et al., 2005). However, acidification of the phagosomes containing *L. monocytogenes* occurs and is required for membrane disruption induced by the bacteria (Beauregard et al., 1997). In non-phagocytic cells, phagosomes containing InlA- or InlB-coated latex beads contain not only endosomal-lysosomal markers but also MSF, which is a member of the septin family involved in membrane fusion events (Pizarro-Cerda et al., 2002).

Escape from phagosomes involves principally three bacterial proteins, the pore forming toxin, LLO (Gaillard et al., 1987), the phosphatidylinositol (PI) phospholipase (PLC-A) (Camilli et al., 1993), and the phosphatidylcholine (PC) phospholipase C (PLC-B) (Grundling et al., 2003). It was suggested that lipoproteins might also participate in the bacterial escape from the phagosome (Reglier-Poupet et al., 2003a).

Once free in the cytosol, *L. monocytogenes* starts multiplying, with an approximate doubling

time of one-hour (Tilney and Portnoy, 1989; Fig. 12C). This is true for most cells studied, but certain listericidal macrophages do not permit intracellular growth, especially when phagocytosis occurs through the C3bi complement receptor CR3 (Drevets et al., 1993). Several genes encoding virulence or metabolic determinants are induced during *L. monocytogenes* intracellular life, including those involved in phagosomal lysis, actin-based motility, and cell-to-cell spreading (Klarsfeld et al., 1994; Freitag et al., 1999; Bubert et al., 2000). Unlike most bacteria, *L. monocytogenes* replicates in the cytosol when it is directly microinjected into cells (Goetz et al., 2001). The cytosol permissiveness for *L. monocytogenes* growth is probably due to its ability to use a variety of cytosolic nutrients, as suggested by the fact that intracellular multiplication of several auxotrophic mutants is not affected (Marquis et al., 1993). Intracytosolic growth of *L. monocytogenes* is dependent on the hexose phosphate transporter *hpt* gene. Expression of *htp* is under the control of the transcriptional activator PrfA (positive regulatory factor A), which regulates most virulence genes (Goetz et al., 2001).

Actin-Based Motility

Upon escape from the phagosome, *L. monocytogenes* gets surrounded by actin filaments and actin binding proteins. The bacteria start moving in the cytosol, where they induce the formation of an actin tail at their rear end, which resembles a comet constantly depolymerizing at its distal end (Tilney and Portnoy, 1989; Cossart and Kocks, 1994; Figs. 12D, 15 and 16). *Listeria monocytogenes* comets are composed of a dense meshwork of cross-linked actin filaments, similar to that induced by *Shigella flexneri* but different from that induced by *Rickettsia conorii*, which are composed of unbranched actin filaments (Gouin et al., 1999, 2005) (Fig. 16). Actin tails are on average 5 µm but can be as long as 40 µm (Tilney and Portnoy, 1989). *Listeria monocytogenes* motility results from the polymerization of cellular actin by the ActA protein expressed at one pole of the bacterial body (Tilney and Portnoy, 1989; Domann et al., 1992; Kocks et al., 1992). The speed of moving bacteria correlates with the length of the comet and is 0.25 µm/s on average. The process of actin polymerization is very dynamic, since the actin tail is constantly polymerizing and depolymerizing, generating the forces to push the bacteria forward (Theriot et al., 1992). Occasionally, propelled bacteria encounter a membrane and form a protrusion (Fig. 12E). When it occurs in the vicinity of an adjacent cell, it allows bacterial dissemination from cell to cell without
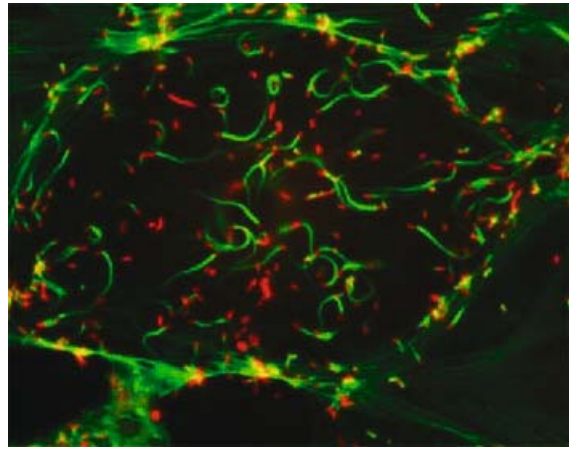
Fig. 15. Actin comets formed in PTK2 after infection with *L. monocytogenes*. PTK2 cells are infected with *L. monocytogenes* for 5 h and fixed. Actin is stained with fluorescein-phalloidin (green) and *L. monocytogenes* are labeled with an anti-*L. monocytogenes* antibody detected by a secondary rhodamine-labeled antibody (red). From N. Khelef, unpublished image.
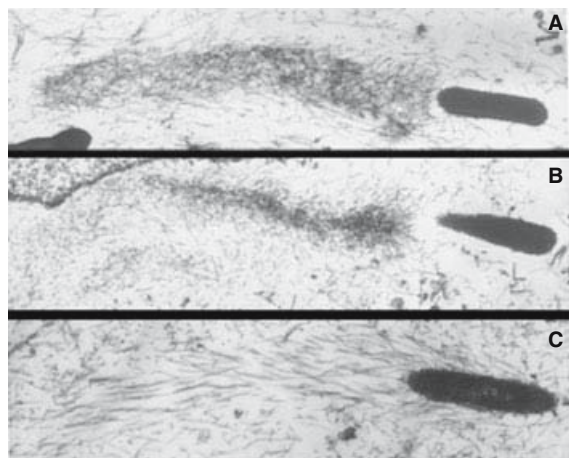
Fig. 16. Electron micrograph of myosin S1 decorated actin comets formed by *L. monocytogenes* (A), *Shigella flexneri* (B) and *Rickettsia conorii* (C) in Hep-2 cells. Adapted from Gouin et al. (1999), with permission.

reaching the extracellular milieu, therefore avoiding the humoral immune response (Tilney and Portnoy, 1989) (Fig. 12F). The actin tail is composed of actin and actin binding proteins. These include the Arp2/3 complex, which is involved in actin nucleation and polymerization (Welch et al., 1997), and VASP, the vasodilator-stimulated phosphoprotein, which directly binds actin (Chakraborty et al., 1995). The actin tail also colocalizes with proteins involved in actin depolymerization, such as cofilin (David et al., 1998; Gouin et al., 1999), or the actin-bundling protein α-actinin (Dabiri et al., 1990) (Fig. 17). The process of *L. monocytogenes* actin based motility involves an Arp2/3-dependent nucleation step and an Arp2/3-independent step,
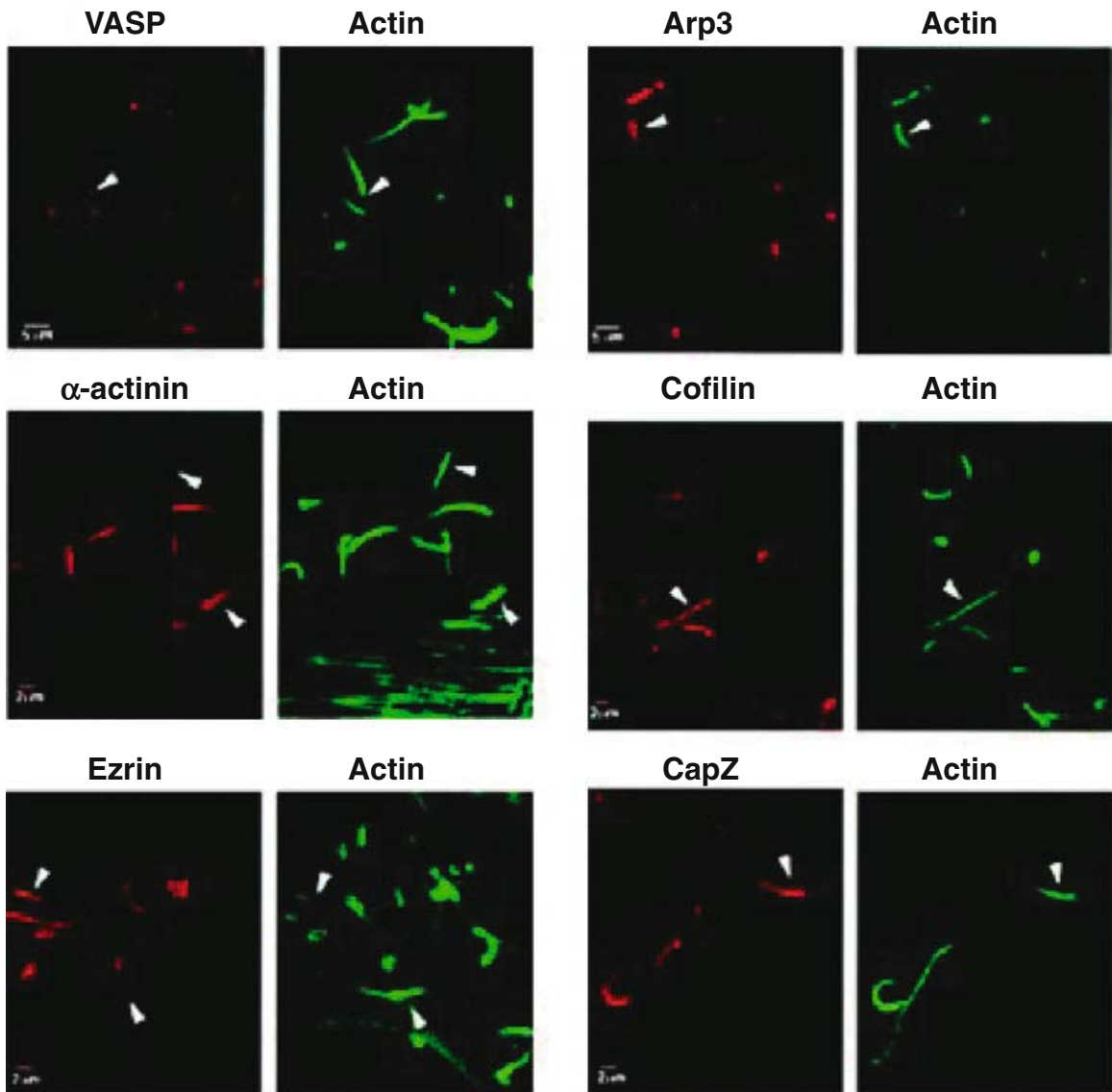
Fig. 17. Localization of the actin partners during the formation of comets in cells infected by *L. monocytogenes*. Actin is labeled with fluorescein isothiocyanate-phalloidin or bodipy-phallacidin (green). Vasodilator-stimulated phosphoprotein (VASP), α-actinin, ezrin, actin-related protein Arp3, cofilin and capZ (capping protein Z) are labeled using specific antibodies detected with secondary fluorescent antibodies (red). Images are acquired by confocal scanning microscopy. From Gouin et al. (1999), with permission.

which requires fascin, an actin bundling protein (Brieher et al., 2004). In addition, it was shown that PI-3K plays a role in *L. monocytogenes* action motility (Sidhu et al., 2005). Using green fluorescent protein fusions proteins (pleckstrin homology domains of phospholipase C and of Akt, respectively), which bind PI(4,5)P2 and PI(3,4,5)P3 or PI(3,4)P2, to detect these phosphoinositides, it was shown that phosphoinositides are recruited around the moving bacteria and then concentrate at the comet tail, explaining the involvement of PI3-K in *L. monocytogenes* motility (Sidhu et al., 2005). The actin-based motility of *L. monocytogenes* is a good example

of the exploitation of the host cell machinery by pathogenic bacteria to promote their own survival (Cossart, 1997). Moreover, this model was efficiently exploited for a better comprehension of complex cellular processes such as the formation of the leading edge during cell migration (Machesky, 1997; Marx, 2003).

Intercellular Spreading

During intracellular multiplication within primary infected cells, *L. monocytogenes* generates an actin comet tail, which propels the bacteria in random directions and occasionally to the cell
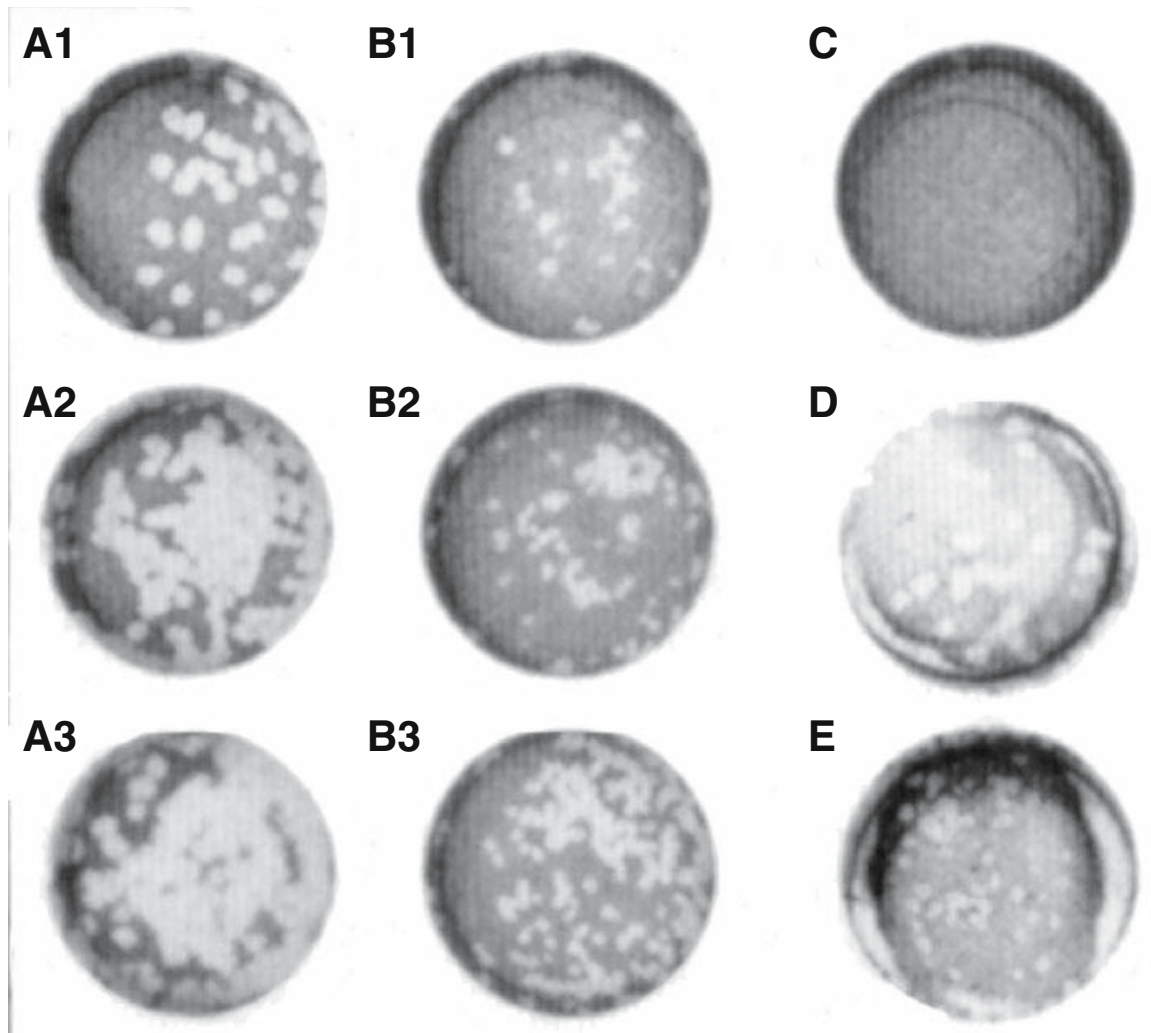
Fig. 18. Plaque formation in 3T3 fibroblasts produced by *L. monocytogenes* infection. Cells were infected with increasing amounts (A1 to A3 and B1 to B3) of *L. monocytogenes* wild-type or *plcB* mutant, or with similar amounts of *L. monocytogenes* wild-type (D), *actA* mutant (C) or *actA* complemented mutant (E). Plaques were visualized by Giemsa staining after 3 days infection. Adapted from Kocks et al. (1992), with permission.

periphery, generating a protrusion, which contains the bacteria. If the moving bacterium encounters the membrane of an adjacent cell, the protrusion invaginates in this cell, generating a double membrane vacuole containing *L. monocytogenes* and part of the actin tail (Fig. 12F and G). The vacuole of the secondarily infected cell is then lysed liberating bacteria into the cytosol and starting a new infectious cycle (Tilney and Portnoy, 1989). Lysis of the double membrane requires both listeriolysin O (LLO) and the phosphatidylcholine phospholipase C (PLC-B), which is matured by the metalloprotease, Mpl (Mengaud et al., 1991c; Domann et al., 1999; Vazquez-Boland et al., 1992; Smith et al., 1995a). The direct cell-to-cell spreading of *L. monocytogenes* can be followed in a plaque formation assay. In this assay, a fibroblast monolayer is infected, covered by an agarose layer containing

gentamicin, which kills extracellular bacteria. After a few hours, plaques corresponding to islets of cells killed by the bacteria can be visualized by Giemsa or crystal violet staining (Kocks et al., 1992; Brouqui et al., 1994; Fig. 18). Their size is proportional to the bacterial ability to infect adjacent cells and disseminate in the monolayer (Havell, 1986). At the molecular level, it was shown that ezrin, a membrane-cytoskeleton linker of the ERM (ezrin-radixin-moesin) family, accumulates at *Listeria* protrusions. Interaction of ERM proteins with membrane components and actin is required for the formation of protrusions and for efficient cell to cell spread (Pust et al., 2005). The *L. monocytogenes* protein ActA plays a key role in cell-to-cell spread since it is required for the efficient actin polymerization responsible for the bacterial motility (Kocks et al., 1992; Cossart and Bierne,

2001). Cell-to-cell spread is an important feature of *L. monocytogenes* pathogenicity since mutants unable to spread from cell to cell is strongly attenuated (Barry et al., 1992). This ability to spread from cell to cell allows bacterial dissemination through an epithelial tissue without reaching the extracellular medium and its antibacterial products including antibodies.

Virulence Factors

Several proteins produced by *L. monocytogenes* are involved in virulence, either in animal or in cellular models. Most of them were identified by transposon mutagenesis in the 1990s (Gaillard et al., 1986; Gaillard et al., 1991; Kathariou et al., 1987; Cossart et al., 1989b; Mengaud et al., 1991b; Kocks et al., 1992). Recently, new techniques, including signature tagged mutagenesis and comparative genomics between pathogenic *L. monocytogenes* and nonpathogenic *L. innocua* isolates, led to the identification of new virulence factors or putative ones (Autret et al., 2001; Glaser et al., 2001; Cabanes et al., 2002, 2004; Dussurget et al., 2002, 2004a; Dramsi et al., 2004; Mandin et al., 2005).

The best characterized virulence factors of *L. monocytogenes* are involved in the four steps of the cell infectious cycle. Entry into epithelial cells is mediated by the internalins InlA and InlB (Gaillard et al., 1991; Dramsi et al., 1995). Lysis of the primary vacuole involves LLO, PLC-A and PLC-B (Gaillard et al., 1987; Camilli et al., 1993; Grundling et al., 2003). Intracellular motility is promoted by the ActA protein (Domann et al., 1992; Kocks et al., 1992). Intercellular spreading involves the ActA protein, LLO, PLC-B, and Mpl, which activates PLC-B (Mengaud et al., 1991c; Kocks et al., 1992; Vazquez-Boland et al., 1992; Smith et al., 1996). Expression of these virulence factors is controlled by the transcriptional regulator PrfA (Kreft and Vazquez-Boland, 2001).

Internalins

Internalins belong to a multigenic family of proteins characterized by a N-terminal domain containing several successive LRR of 22 amino acids. They harbor a signal peptide and are therefore exported at the cell surface (Gaillard et al., 1991; Dramsi et al., 1997; Engelbrecht et al., 1998a; Raffelsbauer et al., 1998). Nine internalins were first identified in the strain EGD (InlA to InlH and InlC2) (Gaillard et al., 1991; Dramsi et al., 1997; Engelbrecht et al., 1998a; Raffelsbauer et al., 1998). The determination of the genome sequence of the strain EGDe then revealed the presence of larger numbers of inter-

nalin or internalin-like genes in the strain EGDe (Cabanes et al., 2002) (Fig. 19). The internalin family can be divided into three classes. The first class comprises internalins containing a LPXTG motif in their C-terminal region (LPXTG proteins), through which they are covalently anchored to the cell wall (Fig. 20). InlA is the best characterized member of this protein family, which contains 18 other members in strain EGDe (Fig. 19A). The second class comprises one internalin in strain EGDe, InlB, which contains a C-terminal region of 80 amino-acid repeats starting with the dipeptide GW modules (Fig. 19B). GW modules mediate a loose association of InlB to the bacterial surface (Fig. 20). A third class is composed of internalins that do not display any surface targeting domain and are therefore secreted in the extracellular medium. InlC is the best characterized member of this family (Fig. 19C). The two first groups of internalins also contain a region of repeated sequences, named B repeats (Vazquez-Boland et al., 2001; Cabanes et al., 2002).

The first internalins to be discovered, InlA and InlB, are the major effectors of *L. monocytogenes* entry into nonphagocytic cells (Gaillard et al., 1991; Dramsi et al., 1995). The functions of the other internalins remain unclear. Single or multiple deletions of internalin genes, including *inlC*, *inlC2-inlD-inlE* (in strain EGD) or *inlG-inlH-inlE* (in strain EGDe), decreased the virulence of *L. monocytogenes* but did not affect entry into epithelial cells or intracellular multiplication (Domann et al., 1996; Dramsi et al., 1997; Raffelsbauer, 1998). This suggests a role for one of these genes in pathogenicity besides invasion. Deletion of the *inlF* gene has no effect on bacterial entry and virulence (Dramsi et al., 1997). A recent study examining double mutants affecting different internalin genes, including, *inlA*, *inlB*, *inlG*, *inlH* and *inlE*, showed that efficient InlA-dependent entry requires the functions of InlC and InlG-InlH-InlE, suggesting a cooperation between the various internalins for efficient cell invasion (Bergmann et al., 2002), as previously shown for InlA and InlB. The presence of multiple internalins suggests that they may also be important for the biology of *L. monocytogenes* besides pathogenesis.

GENETIC ORGANIZATION AND TRANSCRIPTION OF INTERNALIN GENES Internalin genes are dispersed along the chromosome, but some of them are associated in clusters, such as the operon *inlAB* or the clusters *inlG-inlC2-inlD-inlE* or *inlG-inlH-inlE* in *L. monocytogenes* (Vazquez-Boland et al., 2001). Gene rearrangements are suggested by the fact that one internalin locus is different in two other *L. monocytogenes* EGD isolates. Indeed, comparison of the *inlG-inlC2-*

A

B

C
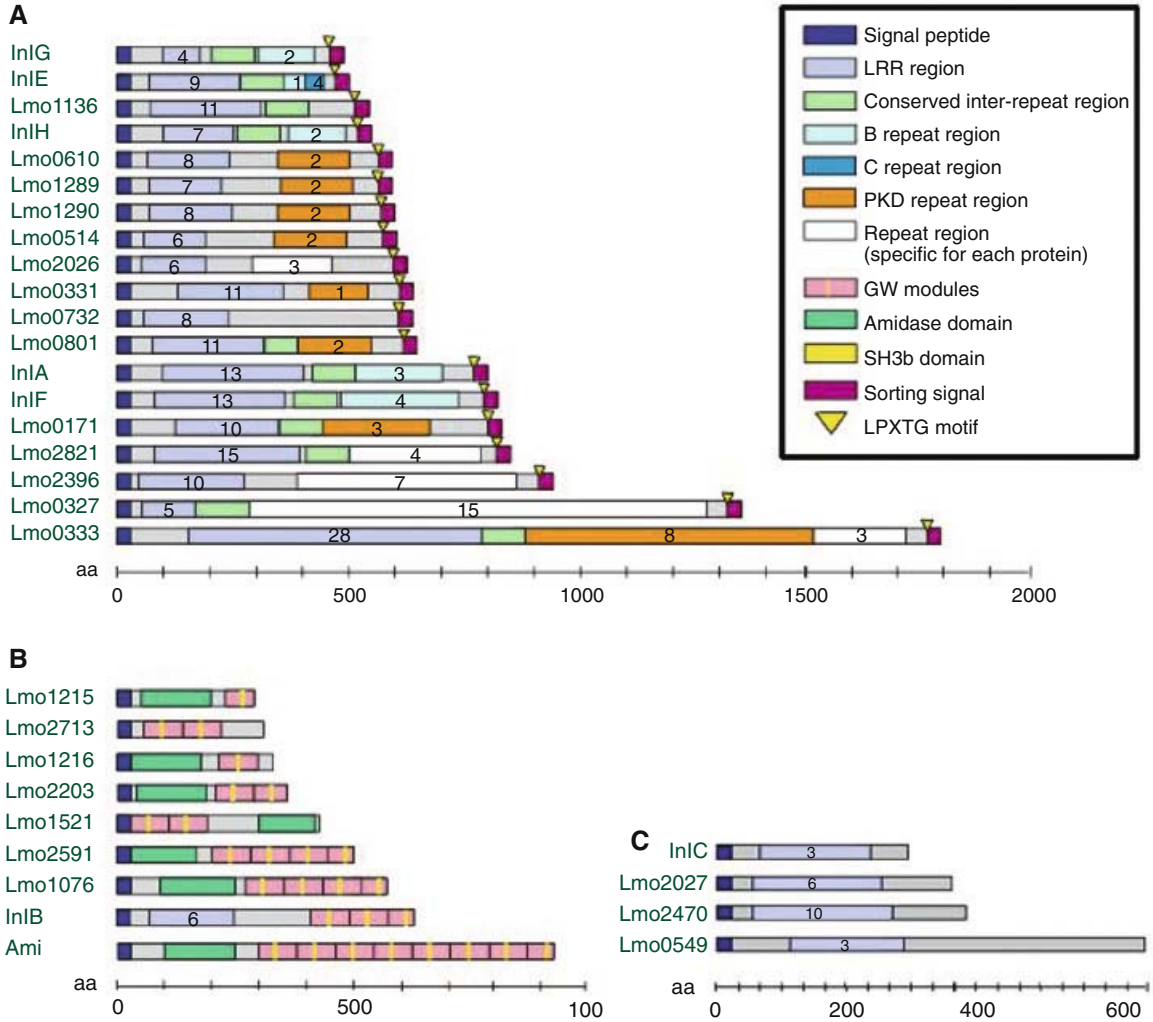
Fig. 19. Alignment of internalin-like LPXTG proteins (A), GW proteins (B), and InlC-like proteins (C). Determined from the annotation of the *L. monocytogenes* genome sequence. The numbers within domains represent the number of repeats. From Cabanes et al. (2002), with permission.
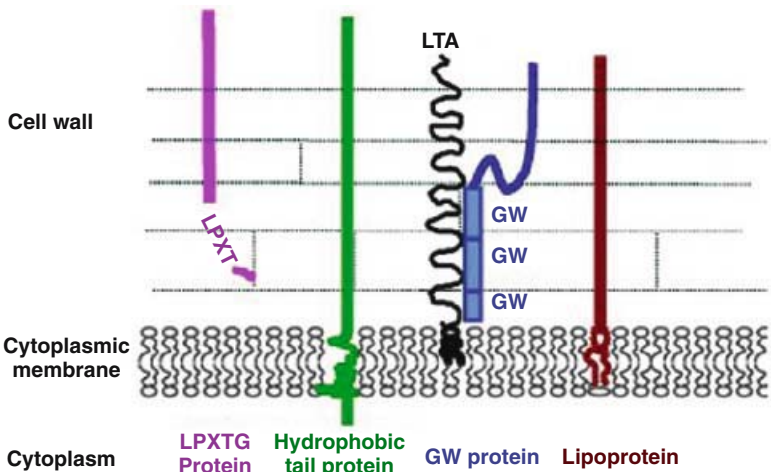
Fig. 20. Association of *L. monocytogenes* surface proteins. Some surface proteins are covalently anchored to the cell wall through a LPXTG motif or loosely attached to the LTAs (lipoteichoic acids) through GW domains. Others are anchored to the cytoplasmic membrane through a hydrophobic tail or a lipid anchor (lipoproteins). From Cabanes et al. (2002), with permission.

Fig. 21. Model for cell wall sorting. The precursor is exported from the cytoplasm via an N-terminal signal sequence (1). The protein is transiently retained in the cytoplasmic membrane by its charged tail and hydrophobic domain (2) allowing recognition and processing of the LPXTG motif by sortase A between the threonine and the glycine residues (3). Sortase A catalyzes the formation of an amide bond between the carboxyl group of threonine and the amino group of the *m*-diaminopimelic acid (DAP) of the cell wall peptidic cross-bridges within a peptidoglycan precursor (4). The precursor is then incorporated into the cell wall by transpeptidation and transglycosylation reactions (5). Adapted from Navarre and Schneewind (1999), with permission.
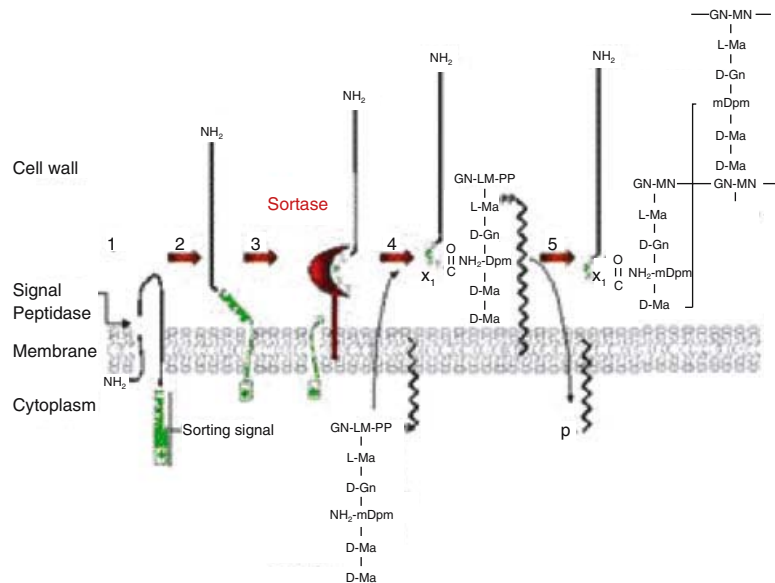
inlD-inlE locus of strain EGD and *inlG-inlH-inlE* locus of another isolate of strain EGD showed that the *inlH* gene probably resulted from the rearrangement between *inlC2* and *inlD* (Dramsi et al., 1997; Raffelsbauer et al., 1998). The *inlA* and *inlB* genes can be transcribed individually or cotranscribed as an operon (Lingnau et al., 1995; Dramsi et al., 1997). Transcription of the *inlA* gene occurs from three promoters, but only one of them is controlled by the transcriptional activator PrfA, which regulates several virulence factors. This PrfA-dependent promoter also controls *inlB* gene expression (Lingnau et al., 1995; Sheehan et al., 1995; Dramsi et al., 1997). Several internalins (including InlA, InlB, InlC2, InlD and InlE) are also positively regulated by the sigma B factor (Kazmierczak et al., 2003). Transcription of the *inlC* gene is also strongly dependent on the PrfA regulator (Engelbrecht et al., 1996), while the *inlGHE* genes are transcribed individually from a major PrfA-independent promoter (Raffelsbauer et al., 1998).

ANCHORING OF INTERNALINS The various surface proteins of *L. monocytogenes* are associated to the bacterial surface, by interacting with either the plasma membrane, peptidoglycan, or cell wall secondary polymers (Fig. 20). In Gram-positive bacteria, anchoring of LPXTG proteins is mediated by sortase A (SrtA), which catalyzes the covalent linkage of the LPXTG motif to the peptidoglycan, after cleavage of the T-G bond of this motif (Mazmanian et al., 1999; Mazmanian et al., 2001; Navarre and Schneewind, 1999) (Fig. 21). In *L. monocytogenes*, deletion of the SrtA gene abolishes the anchoring of most peptidoglycan associated proteins, including InlA (Bierne et al., 2002b; Garandeau et al., 2002). In the

mouse model of infection, after injection by intravenous or oral routes, the virulence of a SrtA-deficient mutant is attenuated, as compared with the isogenic parental strain and with an InlA-deficient mutant. This suggests that LPXTG proteins other than InlA are required for full virulence in this animal model (Bierne et al., 2002b). InlB is not covalently anchored to the peptidoglycan by a LPXTG motif but instead possesses a 232-amino acid region made of two long tandem repeats starting with the dipeptide GW (Fig. 20). These GW modules are responsible for the loose association of InlB to the bacterial membrane through direct interaction with the lipoteichoic acids (LTAs). In addition, the GW modules of InlB interact with two host cell receptors, the glycosaminoglycans (GAGs) and the complement receptor gC1qR (Braun et al., 1997; Jonquieres et al., 1999; Braun et al., 2000; Marino et al., 2002). InlC and InlC-like proteins lack the LPXTG motif, as well as the B repeat region, and are therefore secreted by *L. monocytogenes* (Engelbrecht et al., 1996).

LRRS MOTIFS OF INTERNALINS The LRRs motifs are often involved in protein-protein interactions. This was demonstrated in the interaction of both InlA and InlB with their specific cellular receptors, respectively the human E-cadherin (Lecuit et al., 1997) and the Met receptor (Shen et al., 2000). In prokaryotes, LRR motifs are present in different virulence factors (Table 5). In eukaryotes, LRR proteins are involved in protein-protein interactions in various cellular processes, including recognition of bacterial pathogens by the Toll-like receptors and Nod proteins (Barton and Medzhitov, 2003; Chamaillard et al., 2003), as well as in plant pathogen defenses (Rathjen and Moffett, 2003).

Table 5. Virulence factors containing LRR motifs.

Names	Pathogens	Involved in	References
FHA	*Bordetella pertussis*	Adhesion	Makhov et al., 1994
YopM	Yersiniae	Cellular kinase activation	Leung and Straley, 1989
			McDonald et al., 2003
IpaH	*Shigella flexneri*	Phagosomal escape	Hartman et al., 1900
Slr	Group A *Streptococcus*	Adhesion and phagocytosis	Reid et al., 2003
SlrP	*Salmonella typhimurium*	Host adaptation	Tsolis et al., 1999
SspH2	*Salmonella typhimurium*	Cytoskeleton binding protein	Miao et al., 2003
SspH1	*Salmonella typhimurium*	Inhibition of NF-κB pathway	Haraga and Miller, 2003

Abbreviations: LRR, leucine-rich repeats; FHA, filamentous hemagglutinin; YopM, *Yersinia* outer protein M; IpaH, invasion plasmid antigen; Slr, streptococcal leucine-rich; Ssp, salmonella serine protease.

This similarity could be used as a potential molecular mimicry of the host cell system by the microorganisms.

CELLULAR SPECIFICITY OF INLA AND INLB Both InlA and InlB promote invasion of nonphagocytic cells when coated onto latex beads or expressed by noninvasive bacteria, demonstrating that they are both sufficient for entry (Lecuit et al., 1997; Braun et al., 1998). However, they have different cell specificity, probably due to the presence of their respective receptors on different cell types. InlA mediates entry into cells expressing its receptor, the human E-cadherin, as first shown in vitro for the human enterocyte-like epithelial cell line, Caco-2, and for certain hepatocyte cell lines, such as HepG-2, or placental cells (Dramsi et al., 1995; Mengaud et al., 1996; Lecuit et al., 1997; Lecuit et al., 2004; Bakardjiev et al., 2004). Studies on InlA-dependent entry are now often performed using embryonic fibroblast cells L2071 transfected with the human E-cadherin (Lecuit et al., 2000). In contrast, InlB promotes invasion into several cell types, including certain epithelial cell lines (HeLa, Hep-2 and Vero), hepatocytes (HepG2 and TIB73), brain microvascular endothelial cells (HBMEC), endothelial cells (HUVEC) and fibroblasts (Dramsi et al., 1995; Lingnau et al., 1995; Gregory et al., 1997; Greiffenberg et al., 1998; Parida et al., 1998). Entry through InlA and InlB-dependent pathways were shown to be both dependent on plasma membrane cholesterol, although at different molecular steps (Seveau et al., 2004).

INTERACTION OF INLA WITH ITS RECEPTOR E-CADHERIN AND CONSEQUENCES InlA is the major factor required for *L. monocytogenes* entry into the human enterocyte-like epithelial cell line Caco-2 (Gaillard et al., 1991; Dramsi et al., 1995). When coated onto beads or expressed in noninvasive bacteria, InlA is sufficient to promote invasion of nonphagocytic cells expressing cadherin. Structure function studies showed that the LRRs and the inter-repeat regions of InlA are

sufficient to promote entry into epithelial cells (Lecuit et al., 1997).

Interaction with E-cadherin The receptor for InlA was identified by affinity chromatography as the human E-cadherin, an intercellular adhesion protein, which is highly expressed at the basolateral membrane, in adherens junctions, and possibly on the apical membrane of polarized epithelial cells (Mengaud et al., 1996). E-cadherin is also expressed in hepatocytes, microvascular endothelial cells and choroid plexus cells in the CNS, cytotrophoblastic cells at the placental level, and dendritic cells, suggesting that InlA may target these cells during infection. Interaction of InlA with E-cadherin is the key step in bacterial entry into epithelial cells and in the formation of the phagocytic cup at the entry site. The extracellular domain of E-cadherin interacts with the LRR domain of InlA. *Listeria monocytogenes* recruits α-catenin at its entry site to create a link between E-cadherin and the cytoskeleton through β- and α-catenins (Lecuit et al., 1999; Lecuit et al., 2000) (Fig. 22). Vezatin mediates the bridge between the cadherin-catenins complex and the unconventional myosin VIIa (Kussel-Andermann et al., 2000). Vezatin and myosin VIIa are recruited to the *L. monocytogenes* entry site (Fig. 23) and are involved in the cytoskeleton rearrangements required for efficient InlA-dependent phagocytosis of beads or bacteria (Sousa et al., 2004) (Fig. 24).

Specificity of the InlA-E-cadherin Interaction The InlA-E-cadherin interaction is species specific, since InlA recognizes the human E-cadherin but not the mouse E-cadherin. This specificity is due to a single amino acid, a proline located at position 16 in the binding site of human E-cadherin, which is a glutamic acid residue in the mouse or rat E-cadherins (Lecuit et al., 1999). The completion of the tridimensional structure of the InlA-cadherin complex revealed that the LRR domain of InlA forms a cavity, which is filled by the N-terminal domain of the
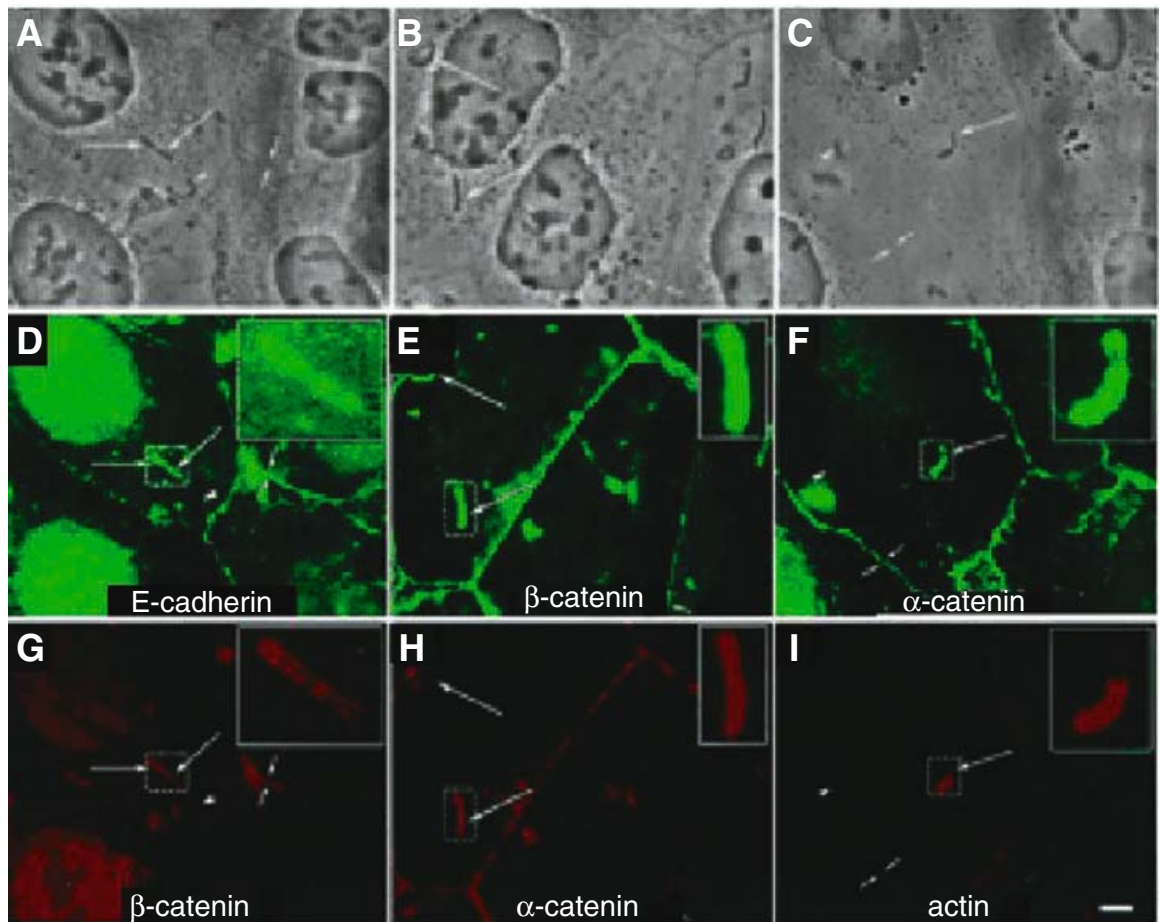
Fig. 22. Recruitment of human E-cadherin, β-catenin, α-catenin and actin during InlA-dependent entry. Caco-2 cells infected with *L. innocua* expressing InlA are observed by phase contrast (A, B and C) or by immunofluorescence microscopy after labeling of actin with fluorescent phalloidin (I) or with E- cadherin (D), β-catenin (E and G) and α-catenin (F and H) antibodies detected using secondary fluorescent antibodies. Arrows and arrowheads indicate sites where protein are or are not recruited around bacteria, respectively. Scale bars, 1 μm. From Lecuit et al. (2000), with permission.

human E-cadherin (Fig. 25). Access of E-cadherin to this pocket is blocked when the proline 16 is changed to a glutamic acid (Fig. 26), promoting a steric hindrance, which abolishes the interaction between the two molecules (Schubert et al., 2002).

Role of InlA in Virulence Before the discovery of the species specificity of the E-cadherin-internalin interaction, no role in virulence could be demonstrated for InlA using the mouse model (Gaillard et al., 1996; Gregory et al., 1996a; Pron et al., 1998). The recent demonstration of the specificity of InlA for human E-cadherin explains these data and has allowed further studies. The role of InlA was assessed in a transgenic mouse model, in which the human E-cadherin is expressed at the intestinal level. Infection of these transgenic mice with a wild-type or a Δ*inlA* mutant showed that InlA was important for the crossing of the intestinal bar-

rier (Lecuit et al., 2001b). In guinea pigs, which possess a permissive E-cadherin, infection of the liver, spleen, lymph nodes and small intestine was strongly reduced after oral inoculation with a Δ*inlA* mutant as compared with wild-type *L. monocytogenes* (Lecuit et al., 2001b). These results confirm that, in permissive species, InlA plays a role in the crossing of the intestinal barrier. Recently, a comparative study between *L. monocytogenes* isolates from sporadic or epidemic cases or associated with healthy human carriages revealed that a high proportion of isolates from food expressed a truncated InlA (35%), whereas expression of a truncated internalin is rare in clinical isolates (7%) and notably absent in isolates from maternofetal infections (Jacquet et al., 2004). Interestingly, these isolates with truncated InlA were affected in their invasive ability, as well as in their pathogenic potential in the chick embryo model, suggesting a role for InlA in the establishment of listeriosis
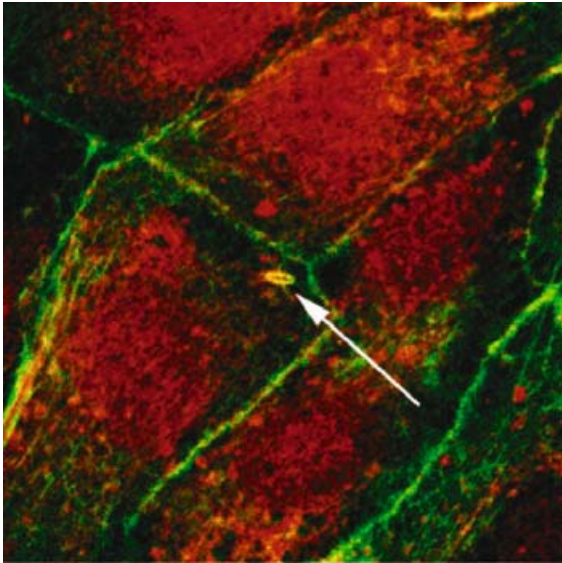
Fig. 23. Recruitment of unconventional myosin VIIa (red) and actin (green) during InlA-dependent entry. Immunofluorescence microscopy images of Caco-2 cells infected with *L. innocua* expressing InlA. Actin is labeled with fluorescent phalloidin. Unconventional myosin VIIa is labeled with specific antibodies detected with secondary fluorescent antibodies. Myosin VIIa is present at the adherens junctions of epithelial cells and is also recruited with actin at the *L. monocytogenes* entry site. Courtesy of Sandra Sousa, unpublished image.
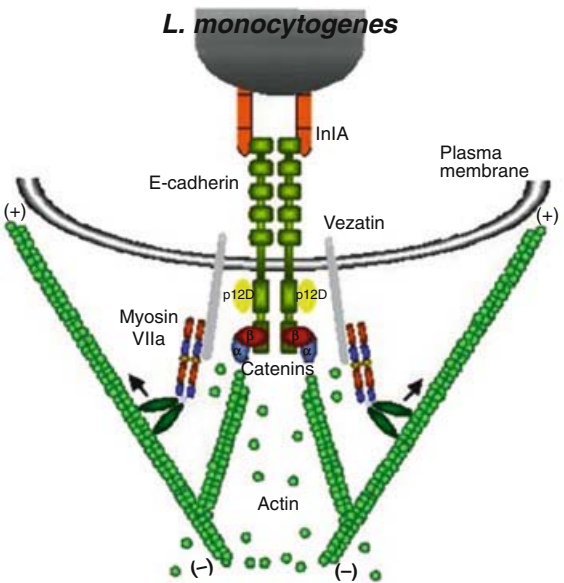
Fig. 24. Model for InlA-dependent entry of *L. monocytogenes* into epithelial cells. Proteins involved in entry are E-cadherin, α- and β-catenins, vezatin, myosin VIIa, and actin. Adapted from Sousa et al. (2004), with permission.
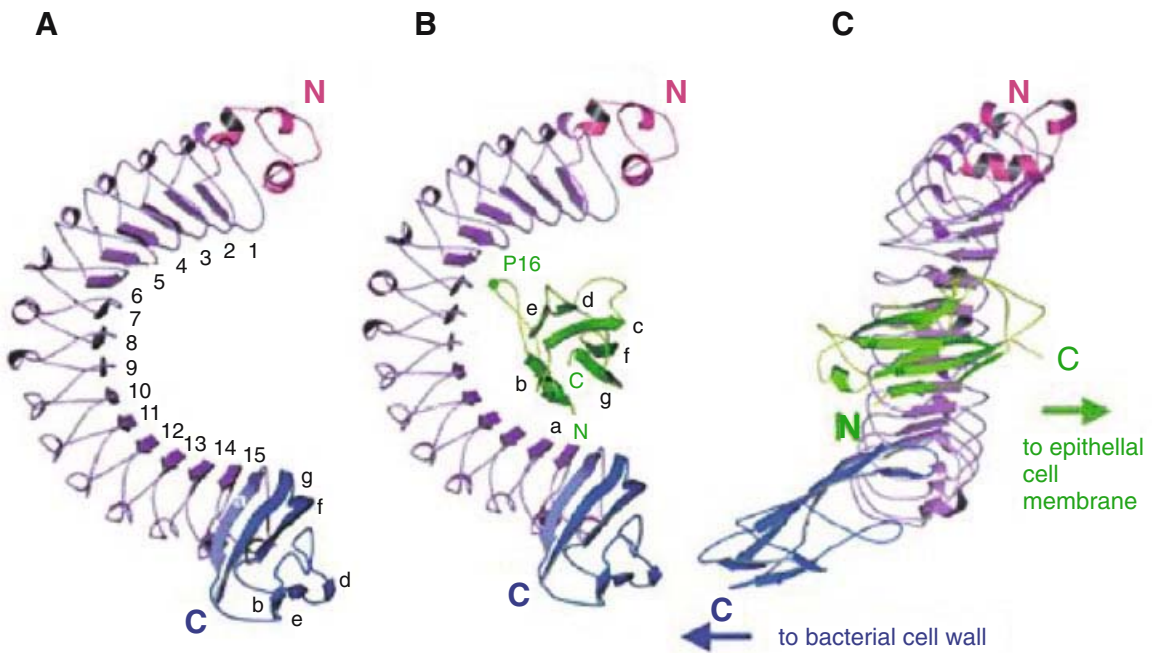
Fig. 25. Model of the three-dimensional structure of InlA' complexed or not with human E-cadherin. A) Uncomplexed InlA. B) InlA' in complex with the EC1 Ig-like extracellular domain of human E-cadherin. C) 90° complex viewed in (B) rotated 90°. InlA' is the functional domain of InlA (residues 36–496). The cap, leucine-rich repeat, and Ig-like inter-repeat domains of InlA' are represented in pink, purple and blue, respectively. The EC1 domain of human E-cadherin containing the crucial proline 16 required for efficient recognition is depicted in green. From Schubert et al. (2002), with permission.
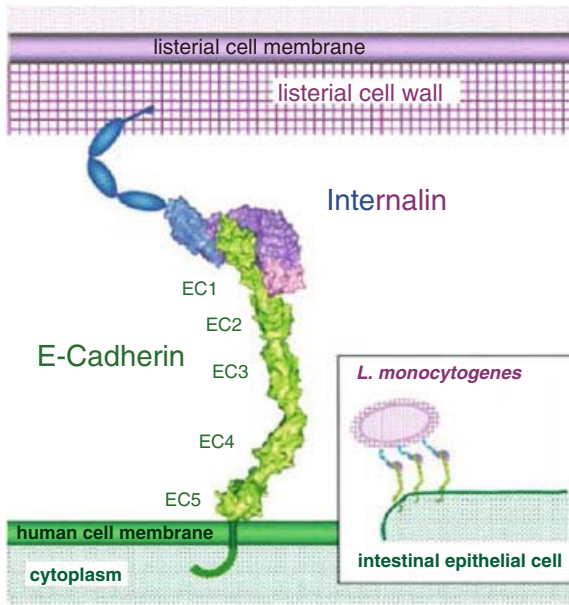
Fig. 26. Representation of the InlA-human E-cadherin complex. InlA, covalently bound to the cell wall of *L. monocytogenes*, binds to the human E-cadherin. EC1 to EC5 are the extracellular domains of human E-cadherin. The cap, leucine-rich repeat and Ig-like inter-repeat domains of InlA' are represented in pink, purple and blue, respectively. From Schubert et al. (2002), with permission.

(Jonquieres et al., 1998; Olier et al., 2003). Exchange of the regions encoding InlA between strains expressing full length or truncated InlA confirmed the requirement for InlA in entry (Olier et al., 2005). The role of InlA in *L. monocytogenes* fetoplacental tropism has now been experimentally addressed. The results show that the ability of *L. monocytogenes* to target the placental villi and cross the placental barrier is dependent upon internalin interaction with trophoblast E-cadherin (Lecuit et al., 2004). Thus, *L. monocytogenes* deploys a common strategy to target and cross the intestinal and placental barriers. This raises the possibility that *L. monocytogenes* placental tropism may be a consequence of its evolved mechanism for targeting the intestinal epithelium. Interestingly, the blood-brain barrier is composed of microvascular endothelium and choroid plexus epithelium expressing E-cadherin. Thus, it is tempting to speculate that *L. monocytogenes* targeting to and invasion of the CNS may also be mediated by the interaction between InlA and E-cadherin.

INTERACTIONS OF INLB WITH ITS RECEPTORS The *inlB* gene was first identified as the second gene of the *inlAB* operon involved in *L. monocytogenes* invasion of epithelial cells (Gaillard et al., 1991). InlB has a role in virulence predominantly

for hepatic colonization, as evaluated in the murine model after intravenous infection (Gaillard et al., 1996; Dramsi et al., 1997). InlB is the major protein required for invasion in a variety of cell types in which InlA plays no role, such as hepatocytes, endothelial cells and fibroblasts (Dramsi et al., 1995; Lingnau et al., 1995; Gregory et al., 1997; Greiffenberg et al., 1998; Parida et al., 1998). InlB is sufficient to promote entry of noninvasive bacteria or beads into nonphagocytic cells (Braun et al., 1998). Soluble InlB induces important cytoskeletal rearrangements (Ireton et al., 1999), generating cell membrane ruffling or cell scattering (Shen et al., 2000). These observations support the hypothesis that InlB, when released from the bacterial surface, may facilitate the disruption of epithelial barriers, allowing dissemination of *L. monocytogenes* to deep tissues. Soluble InlB also stimulates the phagocytosis of noninvasive bacteria, probably as a consequence of cell membrane ruffling (Braun et al., 1998).

Structure and Cell Wall Association of InlB As a member of the internalin multigenic family, InlB possesses a LRR domain and a series of repeats starting with a GW dipeptide at its C-terminus. Both the LRR domain and the GW modules are involved in cell receptor recognition (Braun et al., 2000; Shen et al., 2000; Jonquieres et al., 2001; Marino et al., 2002; Machner et al., 2003). The GW modules of InlB mediate its loose association with the bacterial cell wall through interaction with the membrane-associated LTAs (Braun et al., 1997; Jonquieres et al., 2001) (Fig. 27). However, InlB can also be released in the medium and act as a soluble molecule (Jonquieres et al., 1999). Dissection of the tridimensional structure of the different domains of InlB and of the entire molecule revealed that InlB exhibits an elongated and curved structure, facilitating multiple protein-protein interactions. In addition, the X-ray structure revealed that calcium ions are bound to the N-terminal part of InlB (Fig. 28). Interestingly, the 80-amino acid long GW modules of InlB resemble SH3 domains, although the homology appears rather structural than functional (Marino et al., 1999; Marino et al., 2002).

Interactions of InlB with Its Receptors and Consequences InlB interacts with several cellular receptors, gClqR, Met and GAGs, which probably cooperate to promote bacterial uptake, but their respective roles in the signaling responses to InlB remain to be clarified. The first InlB receptor was identified by affinity chromatography as gC1qR (Braun et al., 2000). gC1qR was initially identified as the receptor for C1q, the first component of the complement cascade
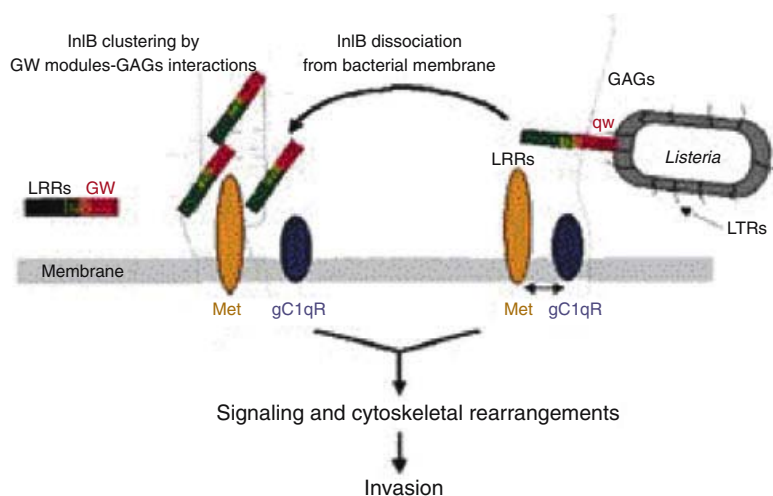
Fig. 27. Model illustrating the synergy between the N- and C-terminal domains of InlB to induce efficient signaling. The GW modules of the C-terminal domain are responsible for the loose association of InlB with the bacterial cell wall lipoteichoic acids (LTAs) and bind to the glycosaminoglycans (GAGs). This interaction can detach InlB from the bacterial surface and allow the interaction of its N-terminal leucine-rich repeat (LRRs) domain with Met, the hepatocyte growth factor receptor. From Jonquieres et al. (2001), with permission.
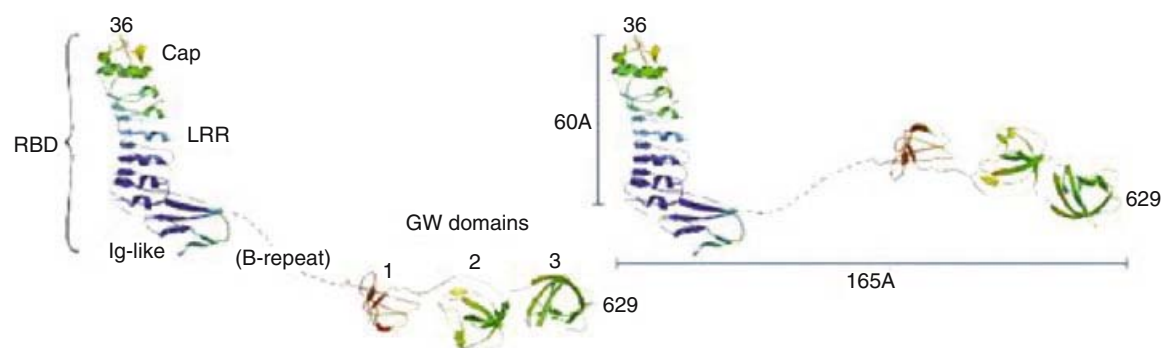
Fig. 28. Three-dimensional structure of InlB. Two different conformers of InlB differing in the path of the B-repeats are represented as ribbons. The red dotted lines represent the B-repeats (not modeled). From Marino et al. (2002), with permission.

(Peerschke et al., 1994). gC1qR is a ubiquitous protein, also called p32. It is present in different subcellular locations and has been involved in various cellular processes, including inflammation and immunity (Ghebrehiwet et al., 2001). InlB-dependent entry is blocked by anti-gC1qR antibodies and by C1q. Moreover, transfection with human gC1qR enhances cell invasion (Braun et al., 2000). These results suggest that gC1qR is an important mediator of bacterial invasion, but since it has neither a transmembrane domain nor a GPI-anchored domain, it was concluded that gC1qR would behave as a co-receptor for a signaling protein. This signaling receptor was later identified as Met, the receptor for the hepatocyte growth factor (HGF). Indeed, Met is a transmembrane protein with a tyrosine kinase activity, which mediates several signaling pathways triggered by InlB. Cells expressing little or no Met are not permissive for InlB-mediated entry and signaling but become permissive when transfected with the human Met receptor (Shen et al., 2000). Both InlB and HGF induce the clathrin-dependent endocytosis and lysoso-mal degradation of Met (Li et al., 2005; Veiga and Cossart, 2005). The Cbl ubiquitin ligase monoubiquitinates Met and is critical for *Listeria* entry into cells (Veiga and Cossart, 2005). InlB also binds directly to cellular GAGs through its GW modules. InlB-dependent entry into epithelial cells is strongly affected by depletion of the cellular plasma membrane GAGs. Finally, heparin, which is a GAG, promotes the detachment of InlB from the bacterial surface and its clustering, suggesting the following model for InlB interaction with its different receptors. Interaction of InlB with GAGs through its GW modules leads to its detachment from the bacterial surface, allowing its clustering at the cellular surface through binding to Met by its LRR domain and favoring the local activation of the signaling pathway downstream of Met (Jonquieres et al., 2001).

Regions of InlB Involved in Receptor Recognition It was shown that the GW modules of InlB interact with gC1qR and GAGs and the LRR domain of InlB binds to Met (Jonquieres

et al., 2001; Marino et al., 2002; Shen et al., 2000). Both regins of InlB, the GW modules and the LRR domain, cooperate for Met activation and InlB-dependent entry (Banerjee et al., 2004).

Species Specificity of InlB As for InlA, InlB was shown to be species specific. It is able to promote entry and ruffling through its Met interaction in humans and mouse cells, but not in guinea pig and rabbit cells, as a problable consequence of Met differences between species (Khelef et al., in press).

InlB-Induced Activation of Phosphoinositide 3-Kinase Cell stimulation with InlB, either soluble or expressed by bacteria, promotes activation of the phosphoinositide 3-kinase (PI3K) (Ireton et al., 1996). This activity strictly depends on the activation of Met. Met recruits and phosphorylates the ubiquitin ligase, Cbl, and the adaptor molecules, Gab1 and Shc, allowing the recruitment of the p85 subunit of type I PI3K to the plasma membrane (Ireton et al., 1999; Shen et al., 2000; Sun et al., 2005). The formation of Gabl-p85 and Gabl-Crk complexes induced by InlB was shown to be important for bacterial entry (Sun et al., 2005). PI3K converts the phosphoinositide PIP2 (phosphatidylinositol 4,5-bisphosphate) into the potent second messenger PIP3 (phosphatidylinositol 3,4,5-trisphosphate), which controls several signaling pathways. In the case of InlB, stimulation of the PI3K through Met activation is critical for phagocytosis, ruffling, and cell scattering, as shown by the inhibitory effects of wortmannin and LY294002, two PI3K inhibitors (Ireton et al., 1996; Ireton et al., 1999; Shen et al., 2000). However, PI3K activation was shown to be dispensable for InlB- or HGF-induced internalization of Met but essential for its lysosomal degradation (Li et al., 2005).

InlB-Induced Phagocytosis Several regulators of actin polymerization and depolymerization are involved in the cytoskeletal rearrangements generated by the interactions of InlB with its receptors have been identified (Bierne and Cossart, 2002a). WASP-related proteins, Abi1, Ena/VASP (Bierne et al., 2005) and the actin nucleator Arp2/3 (Bierne et al., 2001) are recruited to InlB-induced phagocytic cups and membrane ruffles and are required for efficient phagocytosis (Fig. 29). Completion of phagocytosis requires the action of cofilin, which locally depolymerizes actin, allowing the retraction of the cup. Cofilin is recruited to the InlB entry site and accumulates transiently around the nascent phagosome (Fig. 29). The importance of this function was demonstrated by inactivating cofilin, by maintaining it in its inactive form, or by either overexpressing a constitutively activated cofilin

mutant or using a dominant negative mutant of the LIM kinase, which inactivates cofilin. The recruitment of Arp2/3, cofilin, and LIM kinase to the entry site is proposed to occur through the activation of the small GTPase Rac and Cdc42 depending on the cell type (Bierne et al., 2001; Bierne et al., 2005).

InlB-Induced Signaling In addition to its role in phagocytosis, InlB also stimulates other PI3K-dependent pathways, such as the PLC-γ (Bierne et al., 2000), the Ras-Akt-NF-κ B signaling cascade (Mansell et al., 2000; Mansell et al., 2001), and the Ras-MAP kinases pathway (Copp et al., 2003) (Fig. 30). These different pathways have been involved in the regulation of cell survival and anti-apoptosis by growth factors, suggesting that InlB might induce these activities to counteract the deleterious effect of *L. monocytogenes* infection. Since cell membrane ruffling and scattering are also observed after stimulation with InlB and HGF, revealing that InlB behaves as a growth factor (Bierne and Cossart, 2002a).

INTERNALINS IN OTHER *LISTERIA* SPECIES Five internalin genes were first identified in *L. ivanovii* strains, but DNA hybridization experiments showed that internalin-like genes were also present in other *Listeria* species (Gaillard et al., 1991; Dramsi et al., 1997). *Listeria ivanovii* internalins harbor the typical LRR domain. They are small-secreted proteins, while most *L. monocytogenes* internalins are associated with the bacterial cell wall (Engelbrecht et al., 1998b; Vazquez-Boland et al., 2001). Importantly, the two major proteins involved in invasion by the human pathogen *L. monocytogenes*, InlA and InlB, are absent from the animal pathogen *L. ivanovii* suggesting that internalins are critical for Listeriae interactions with their hosts and environment.

Listeriolysin

Listeriolysin O or LLO was the first, and so far the most potent, virulence factor identified in *L. monocytogenes* (Gaillard et al., 1986) (Fig. 4). LLO is a 60-kDa hemolysin belonging to a family of antigenically related cholesterol-dependent pore-forming toxins or cholesterol-dependent cytolysins, which also includes the streptolysin O (SLO) of *Streptococcus pyogenes* and perfringolysin O (PFO) of *Clostridium perfringens* (Gilbert, 2002). Cholesterol-dependent cytolysins are inhibited by oxidation or by thiol-reacting products but reactivated by thiol-reducing compounds (Alouf, 1999). They are characterized by a ECTGLAWEWWR motif, which includes the
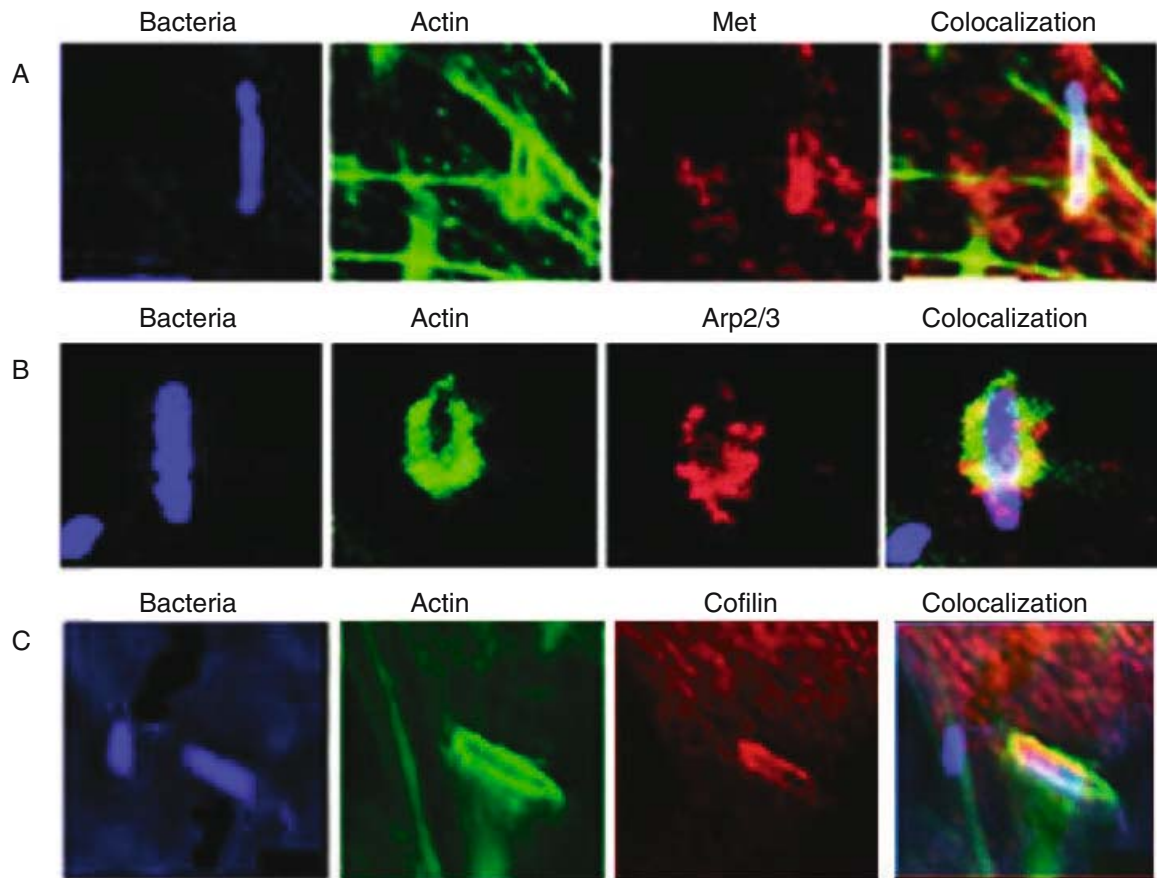
Fig. 29. Recruitment of Met (hepatocyte growth factor receptor), Arp2/3 complex, cofilin and actin during InlB-dependent entry. Immunofluorescence microscopy images of Vero (A and B) or Ref52 (C) cells infected with *L. monocytogenes*. Bacteria appear in blue. Actin is labeled with fluorescent phalloidin (green). Met, Arp2/3 and cofilin are labeled with specific antibodies detected with secondary fluorescent antibodies (red). Colocalization corresponds to overlays of green and red images. From Bierne et al. (2001), with permission.

unique cysteine residue of the LLO. However, mutation of this residue is not essential for hemolysis, while the last Trp residue of the motif (Trp-432) was shown to play a crucial role for full hemolytic activity and virulence (Michel et al., 1990; Gilbert, 2002).

LLO is encoded by the *hly* gene, which was first identified as a factor absolutely required for virulence by analyzing nonhemolytic mutants obtained by transposon mutagenesis (Gaillard et al., 1986; Kathariou et al., 1987). Virulence of the nonhemolytic mutants is strongly attenuated in the mouse model, with a four log difference in the LD_{50} after intravenous inoculation (Gaillard et al., 1986; Kathariou et al., 1987). Nonhemolytic mutants are also affected in both epithelial and phagocytic cellular models of infection, in which they fail to escape from the phagosomes and to multiply intracellularly (Berche et al., 1988; Kuhn et al., 1988). Genetic analysis of these mutants showed that the LLO defect could be complemented by the reintroduction in trans of the *hly* gene (Cossart et al., 1989b). Similarly, spontane-

ous *hly* revertants recover their hemolytic and pathogenic properties (Gaillard et al., 1986), confirming the central role of LLO in these processes.

LLO is required for escape from both the primary vacuole and of the double membrane-bound phagosomes, cooperating with the PLC-A and PLC-B. It is essential for intracellular multiplication (Gaillard et al., 1987; Bielecki et al., 1990; Gedde et al., 2000). It is proposed that the pore forming ability of LLO mediate the lysis of the vacuoles. In addition, LLO is also responsible for important features of *L. monocytogenes* interactions with the host. They include activation of several signaling pathways (Wadsworth and Goldfine, 1999; Repp et al., 2002; Wadsworth and Goldfine, 2002; Dramsi et al., 2003), production of cytokines (Nishibori et al., 1996), induction of apoptosis of dendritic cells and lymphocytes in vitro (Guzman et al., 1996; Carrero et al., 2004) and of lymphocytes during mice infection (Carrero et al., 2004), and development of a protective immune response against *L. monocytogenes* infection (Berche et al., 1987a;
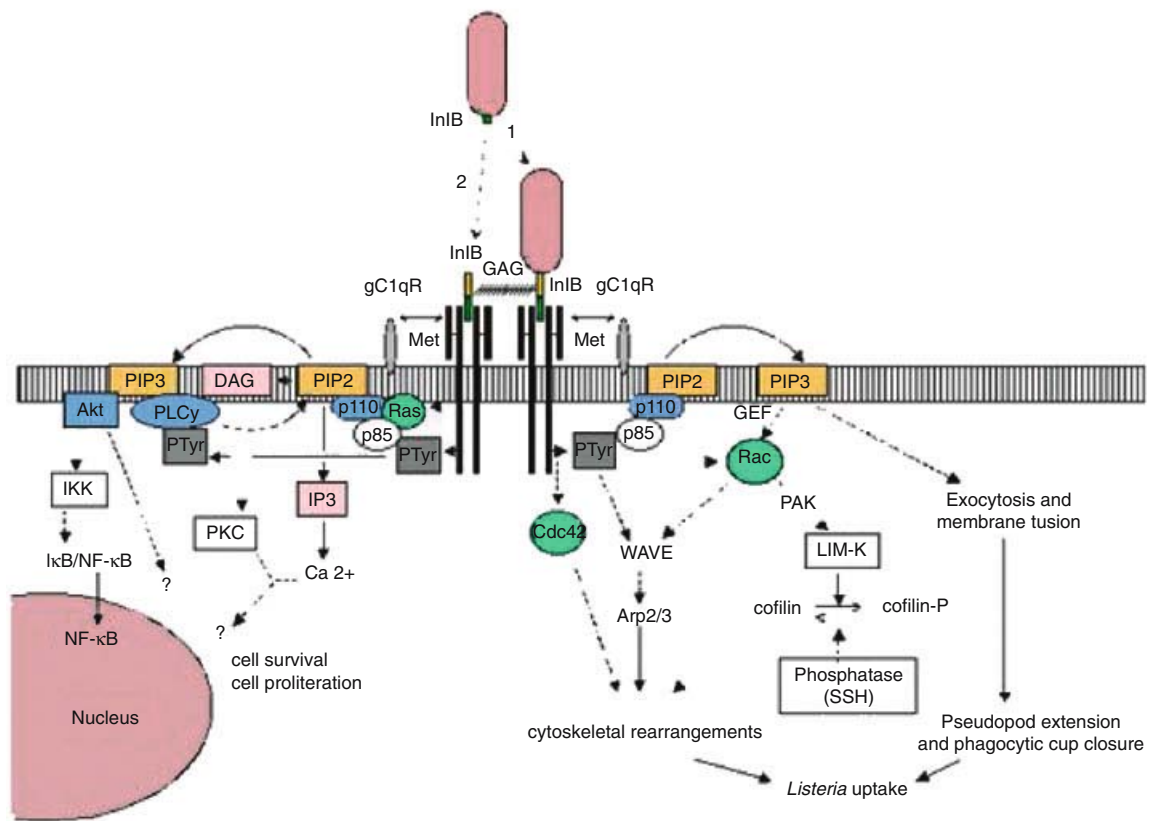
Fig. 30. The InlB-mediated signaling pathway. InlB buried in the bacterial cell wall may dissociate from the bacterial surface by interacting with glycosaminoglycans (GAGs) and possibly gC1qR (C1q complement component receptor), becoming accessible to bind Met (hepatocyte growth factor receptor). The surface-exposed InlB will trigger entry of the bacteria (1) while a pool of soluble InlB released in the medium will signal independently or as a prelude to bacterial entry (2). Interaction of InlB with Met induces the recruitment of adaptor proteins, the recruitment and activation of the phosphoinositide 3-kinase (PI3K). This triggers actin cytoskeletal rearrangements involving Rho-GTPases and cytoskeletal regulatory proteins and membrane reorganization, which lead to bacterial uptake. Activation of other signaling molecules such as phospholipase C γ (PLC-γ) and the kinase Akt might affect the fate of bacteria and/or cells. From Bierne and Cossart (2002a), with permission.

Pamer, 2004). Antibodies specific for LLO are produced during human listeriosis and their detection is used for diagnosis (Berche et al., 1990). Interestingly, because it can lyse phagosomal membranes, LLO (alone or included in liposomes) has been used as an intracytosolic delivery system for antigens, DNA, or antisense oligodeoxynucleotides. This strategy has been exploited to facilitate research progress in gene therapy, cancer treatment, and vaccination (Lee et al., 1996; Dietrich et al., 2001; Mathew et al., 2003; Provoda et al., 2003).

ROLE OF LISTERIOLYSIN The pathogenic potential of Listeriae has been linked to their ability to induce hemolysis, and therefore to the production of LLO. However, despite its fundamental role in virulence, LLO by itself is not sufficient to confer a pathogenic potential to nonpathogenic Gram-positive bacteria (Portnoy et al., 1992). Moreover, *L. seeligeri*, which has weak

hemolytic activity, is rarely pathogenic for humans, suggesting that hemolysin is not the only factor required for *L. monocytogenes* virulence (Seeliger and Jones, 1986; Hof and Hefner, 1988). Heterologous expression of the *hly* gene in *Bacillus subtilis* enabled this organism to lyse primary phagosomes and to multiply intracellularly, confirming the role of LLO escaping from the primary vacuole (Bielecki et al., 1990). When this experiment was performed with the genes encoding PFO or SLO, it was found that only PFO, and not SLO, promoted phagosomal disruption (Portnoy et al., 1992). This shows that despite the high level of homology of Cholesterol-dependent cytolysins, their ability to disrupt the phagosomal membrane is not conserved. Similarly, expression of ivanolysin, the LLO homologue expressed by *L. ivanovii*, can replace LLO to allow vacuolar escape of *L. monocytogenes*. However, it is not sufficient to confer full virulence in mice, as a •*hly L. monocytogenes* mutant expressing ivanolysin colonizes

efficiently the liver but does not persist in the spleen (Frehel et al., 2003).

MECHANISMS UNDERLYING LISTERIOLYSIN ACTIVITY On the basis of structure-function and biochemical studies, as well as the sequence homology between LLO, PFO and SLO, the mechanism of LLO interaction with membranes is currently thought to be as follows. After an initial step of binding to membrane cholesterol, the toxin monomers diffuse laterally to form ring-shaped oligomers inserted in the membrane bilayer, forming ion-permeable pores (30 nm in diameter) without disrupting the plasma membrane (Vazquez-Boland et al., 2001; Repp et al., 2002) (Fig. 31). Optimal hemolytic activity of LLO is observed at acidic pH, as in the case of phagosomes containing *L. monocytogenes*, supporting the idea that LLO is activated inside the phagosome and inactivated in the cytosol, preventing cellular damage (Geoffroy et al., 1987; De Chastellier and Berche, 1994; Beauregard et al., 1997). Interestingly, LLO is degraded at neutral pH, as a consequence of its denaturation, which results from the unfolding of its transmembrane domain. This process also contributes to the control of LLO activity (Schuerch et al., 2005). Permeabilization of the vacuolar membrane occurs as the pH rises, probably resulting from the equilibration with the cytosol (Beauregard et al., 1997). Experimental evidence for the role of LLO in this process was provided using a mutant of *L. monocytogenes* carrying an IPTG-inducible *hly* gene. IPTG induction was performed when bacteria were in the phagosome, resulting in production of active LLO and disruption of the phagosomal membrane that promoted bacterial release in the cytoplasm (Dancz et al., 2002). Disruption of the primary phagosome and of the double membrane vacuole requires the concomitant effect of the LLO and phospholipase activities (PLC-A and PLC-B, respectively) and leads to disruption of the phagosome, releasing *L. monocytogenes* into the

cytosol (Beauregard et al., 1997; Glomski et al., 2002). Interestingly, LLO is not always essential for phagosome disruption, as in certain cell types (HeLa and HenLe 407), the lysis of the vacuole occurs in absence of LLO and is only mediated by PLC-B (Marquis et al., 1995; Grundling et al., 2003).

Interactions of Listeriolysin with Cholesterol Cholesterol is an important factor for LLO binding to plasma membrane. However, preincubation of LLO with cholesterol inactivates its hemolytic activity without blocking its binding to plasma membranes (Geoffroy et al., 1987; Jacobs et al., 1988; Coconnier et al., 2000). Inhibition appears to occur at the step of toxin oligomerization rather than at the initial step of membrane association (Jacobs et al., 1998; Coconnier et al., 2000). In polarized intestinal cells, LLO is specifically targeted to plasma membrane lipid rafts, probably because of its high affinity for cholesterol, and is internalized through caveolae (Coconnier et al., 2000). In agreement with this, LLO was also shown to aggregate several raft-associated molecules, including a signaling molecule, the Lyn kinase, a process which is proposed to mediate LLO signaling ability. This aggregation is independent of the cytolytic activity of LLO and results from the oligomerization of LLO monomers in the membrane (Gekara et al., 2005). In polarized intestinal cells, internalization of LLO through caveolae leads to stimulation of mucus exocytosis in cultured intestinal mucus secreting cells (Coconnier et al., 2000).

pH Dependence of Listeriolysin As compared with other Cholesterol-dependent cytolysins, the main feature of LLO is its optimal activity at acidic pH (Geoffroy et al., 1987). This property has important consequences since LLO is fully active in acidic vacuoles, which allows escape from the phagosome without destroying the plasma membrane (Beauregard et al., 1997). An elegant study has shown that this property is
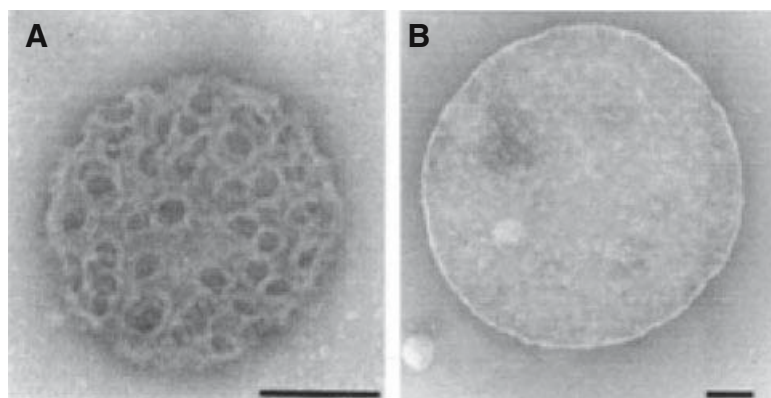
Fig. 31. Electron micrograph of red blood cells after treatment with listeriolysin (A) or cholesterol-inactivated listeriolysin (B). Scale bars, 100 nm. From Jacobs et al. (1998), with permission.

important for completion of *L. monocytogenes* infectious cycle. Indeed, intracellular expression of PFO under the control of the *hly* promoter resulted in lysis of the phagosomal membrane but was followed by cell lysis. Mutations in PFO modifying its optimal pH (from neutral to acidic) abolished its cytotoxicity without affecting its ability to disrupt the phagosome (Jones et al., 1996). Conversely, mutations in LLO modifying its pH optimum (from acidic to neutral) conferred a stronger hemolytic activity but also a lower pathogenic potential. Indeed, a *L. monocytogenes* mutant with a LLO active at neutral pH was able to lyse the primary phagosome and to disseminate by cell-to-cell spreading. It was more cytolytic than the wild-type strain. Its LD_{50} was 100-fold higher in the mouse model (Glomski et al., 2002). This attenuated virulence is probably due to the release of cytolytic bacteria into the extracellular milieu and their exposure to antimicrobial defenses. Similarly, mutants of LLO that fail to compartmentalize its activity were more cytotoxic in vitro but less virulent in vivo (Glomski et al., 2003).

PEST-like Motif of Listeriolysin A recent analysis of the N-terminal domain of LLO revealed the presence of a PEST-like sequence, which is involved in targeting proteins to the proteasome dependent degradation pathway. LLO mutants lacking this sequence were cytotoxic and were affected in virulence (Decatur and Portnoy, 2000; Lety et al., 2001). The presence of this PEST-like motif could appear as another strategy to overcome the toxic potential of LLO by targeting the toxin to the degradation pathway when released in the cytosol. However, this theory is challenged by controversial results concerning the role of the PEST motif. One group showed that deletion of the PEST sequence resulted in LLO accumulation in the cytosol without defect in escape from the phagosome (Decatur et al., 2001). Another group showed that mutations in or near the PEST motif of LLO did not promote accumulation of the toxin in the cytosol but decreased LLO ability to disrupt the phagosome. This suggests that the conformation of the domain surrounding the PEST motif, rather than the PEST sequence itself, is important for this activity (Lety et al., 2002; Lety et al., 2003).

LISTERIOLYSIN-INDUCED SIGNALING IN EUKARYOTIC CELLS LLO is a very potent inducer of cell signaling involved in several biological processes, ranging from crucial features of the infectious process to other signals whose roles remain to be clarified. These include stress responses, cell proliferation, secretion of mucus, immunomodulation, or activation of proinflammatory cascades.

Activation of Second Messengers In epithelial and endothelial cells, the pore forming ability of LLO stimulates a transient influx of extracellular calcium, which is mediated by its pore forming ability (Wadsworth et al., 1999; Rose et al., 2001; Repp et al., 2002; Dramsi et al., 2003). The consequences of this activity include modulation of cellular gene expression, signaling, and modulation of *L. monocytogenes* invasion in epithelial cells. LLO modifies the phosphoinotide metabolism and the generation of lipid mediators, such as diacylglycerol, platelet-activating factor, ceramide, and prostaglandins in endothelial cells or platelet-activating factor and leukotrienes in polymorphonuclear leukocytes (PMN) (Sibelius et al., 1996a; Sibelius et al., 1996b; Sibelius et al., 1999; Rose et al., 2001). LLO also induces PMN degranulation characterized by elastase secretion (Sibelius et al., 1999). In macrophages, LLO induces an increase in phosphoinositide hydrolysis, activation of the host PI-PLC, phospholipase D, and protein kinase C (PKC) α and β (Goldfine et al., 2000; Wadsworth and Goldfine, 2002).

Activation of MAP Kinases and Nuclear Factor Cascades LLO induces several well-known signaling cascades, including the Raf-MEK-MAP kinase pathway (Tang et al., 1996; Tang et al., 1998; Weiglein et al., 1997), the NF-κ B and the AP-1 nuclear factor pathways (Kayal et al., 2002; Lievin-Le Moal et al., 2002), as well as calcium- or lipid-dependent signaling pathways. LLO-dependent activation of MAP kinases in HeLa cells concerns Erk2, p38 MAPK, and c-Jun. Hemolytic activity is required for the activation of MAP-kinase activation and for *L. monocytogenes* invasion of HeLa cells (Tang et al., 1998). LLO-induced activation of NF-κ B is implicated in cytokine production, endothelial cell activation, and mucus secretion (Kayal et al., 1999; Rose et al., 2001; Lievin-Le Moal et al., 2002). Activation of NF-κ B involves the classical inactivation of the NF-κ B inhibitor Iκ B kinase in a process that does not involve the interleukin 1 (IL1) signaling pathway (Kayal et al., 2002).

Activation of Mucus Secreting Cells Interestingly, LLO activates the expression of mucin genes and the exocytosis of mucins by polarized intestinal mucus-secreting cells through activation of NF-κ B and AP-1 transcription factors (Lievin-Le Moal et al., 2002). This process is independent of the pore-forming activity of LLO (Coconnier et al., 2000). Importantly, induction of these activities by LLO results in the inhibition of *L. monocytogenes* entry into mucin secreting cells (Lievin-LeMoal et al., 2005).

Activation of Proinflammatory Cytokines
Among the most important signaling activities mediated by LLO is its ability to induce the expression of a wide range of proinflammatory molecules in both macrophages and endothelial cells (Krull et al., 1997; Rose et al., 2001). LLO, either purified or expressed by *L. monocytogenes*, induces the production of interleukins (IL1 and IL12), tumor necrosis factor α (TNFα) and type I interferon β (IFNβ) by macrophages as well as interferon γ (IFNγ) by natural killer (NK) cells (Yoshikawa et al., 1993; Nishibori et al., 1996; Kohda et al., 2002; Nomura et al., 2002; Stockinger et al., 2002). Interestingly, both IL1 and IFNγ production are independent of the hemolytic activity of LLO (Yoshikawa et al., 1993; Nishibori et al., 1996). LLO also activates endothelial cells by stimulating the production of nitic oxide, adhesion molecules (ICAM–1, V-CAM-1 and E-selectin), chemokines and pro-inflammatory cytokines (MCP-1, IL6, IL8 and GM-CSF), probably through NF-κ B activation (Krull et al., 1997; Drevets, 1998; Kayal et al., 1999; Rose et al., 2001). It has been proposed that, in vivo, the production of IFNγ or TNFα mediate the endothelial cell activation, allowing the recruitment of T cells to the site of infection and contributing to the protective acquired immune response against *L. monocytogenes* (Xiong et al., 1994; Vazquez et al., 1995). In the epithelial cell line Caco-2, membrane permeabilization by LLO was shown to trigger expression of the interleukin IL6, in a Ca^{2+} dependent manner (Tsuchiya et al., 2005).

ROLE OF LISTERIOLYSIN IN THE PROTECTIVE IMMUNE RESPONSE LLO is involved in the induction of a protective immune response against *L. monocytogenes* infection in different ways. LLO is a major protective antigen recognized by cytotoxic CD8^{+} lymphocytes, in agreement with the cellular immune response characterizing *L. monocytogenes* infection (Berche et al., 1987a; Bouwer et al., 1992; Sirard et al., 1997). Moreover, presentation of major histocompatibility complex (MHC)-I restricted antigens of *L. monocytogenes* and induction of a specific protective immune response result from the release of bacteria inside the cytosol and their intracellular growth. Since LLO mediates this process, it is therefore important for an efficient cellular immune response against *L. monocytogenes* (Darji et al., 1995; Darji et al., 1997). However, LLO also elicits a strong humoral immune response, which, in restricted cases, can be protective against *L. monocytogenes* infection (Berche et al., 1990; Gholizadeh et al., 1996; Grenningloh et al., 1997; Edelson et al., 1999). This has led to the reconsideration of the previous thought that protective immunity against *L.*

monocytogenes infection was only cellular and not humoral. Finally, LLO mediates the delivery of soluble antigen to the MHC-I presentation pathway, which is another way of participating in the induction of specific cellular immunity (Berche et al., 1987b; Brunt et al., 1990; Hitbold et al., 1996; Lee et al., 1996).

Phospholipases and Metalloprotease

Two phospholipases are produced by *L. monocytogenes*, PLC-A or PI-PLC and PLC-B or PC-PLC (Mencikova, 1989; Geoffroy et al., 1991; Leimeister-Wachter et al., 1991; Mengaud et al., 1991a; Mengaud et al., 1991c). Another phospholipase SmcL is produced by *L. ivanovii* (Mencikova, 1989; Gonzalez-Zorn et al., 1999). The three phospholipases have a membrane damaging activity and are involved in bacterial escape from primary and/or secondary phagosomes (Vazquez-Boland et al., 1992; Camilli et al., 1993; Marquis et al., 1995; Gonzalez-Zorn et al., 1999; Grundling et al., 2003). Each of the two *L. monocytogenes* phospholipases is important for virulence since mutants deficient in either PLC-A or PLC-B are attenuated (Camilli et al., 1991; Smith et al., 1995). More importantly, double mutants deficient in both phospholipases are 500 times less virulent than single mutants, emphasizing the importance and the complementarity of these factors in listeriosis (Smith et al., 1995).

PHOSPHOLIPASE PLC-A The phospholipase PLC-A is a 33-kDa secreted protein, whose enzymatic activity is specific for PI (PI-PLC) (Mengaud et al., 1991a). PLC-A is produced by *L. monocytogenes* and *L. ivanovii* and is similar to the PI-PLCs of *Bacillus thuringensis*, *Bacillus cereus* and *Staphylococcus aureus* (Leimeister-Wachter et al., 1991; Mengaud et al., 1991a). PLC-A is encoded by the *plcA* gene, which is upstream of the transcriptional activator gene *prfA* and opposite the *hly* gene encoding listeriolysin O (Leimeister-Wachter et al., 1991; Mengaud et al., 1991a; Mengaud et al., 1991b). During early exponential growth, the *plcA* and *prfA* genes are cotranscribed in a 2.2-kb plcA-prfA transcript. During late exponential growth, *plcA* gene expression is monocistronic. PLC-A expression is dependent on the transcriptional activator PrfA (Mengaud et al., 1991b). The PLC-A crystal structure revealed that it consists of a single $(βα)_8$-barrel domain with its active site at the C-terminal side of the β-barrel (Fig. 32). This structure is highly homologous to the *B. cereus* PI-PLC despite the low sequence homology between the two proteins (Moser et al., 1997).

Enzymatic Activities of PLC-A PLC-A is specifically active on PI but not on PC, phospha-
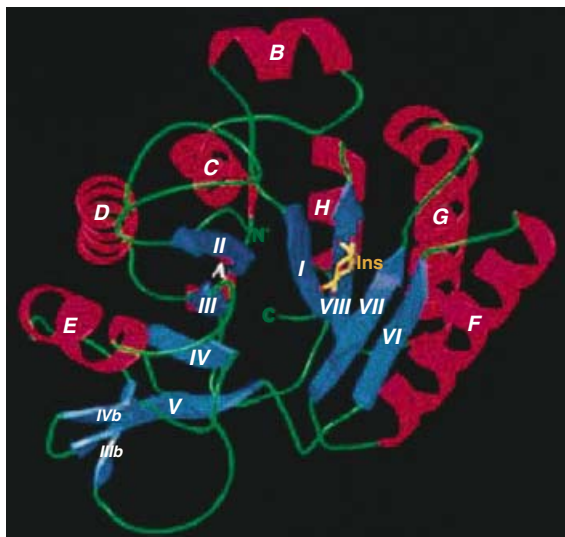
Fig. 32. Three-dimensional structure of phosphatidylinositol phospholipase C (PLC-A). Ribbon diagram of the structure of PLC-A viewing towards the active site pocket with a bound inositol molecule (Ins, bonds in yellow). α-Helices, β-strands and loops are colored in red, blue and green, respectively. From Moser et al. (1997), with permission.

tidylethanolamine or phosphatidylserine (Goldfine and Knob, 1992). It is active on eukaryotic GPI-anchored proteins including those of *Trypanosoma brucei* membranes, although with a low efficiency as compared with *B. thuringensis* PLC (Goldfine and Knob, 1992; Gandhi et al., 1993). PLC-A has a pI of 9.4 and an optimal pH of 5.5–6.5 in Triton X-100 micelles, in agreement with its intraphagosomal activity (Goldfine and Knob, 1992). Its activity is stimulated by salts, such as $CaCl_2$, $MgCl_2$ or KCl, with no specific dependence on divalent cations, and is not inhibited by EDTA (ethylene diamine tetraacetic acid) (Goldfine and Knob, 1992). Using a fluorigenic substrate analog of PI, the enzymatic properties of the PLC-A were analyzed. It was shown that a short-chain phospholipid (diC(6)PC) activates the enzyme in a process fitting with a two-site model, in which the substrate and the activator bind to different sites interacting with each other (Ryan et al., 2002). Mutations of the two potential active-site histidine residues (H38 and H86) revealed that they are both required for the enzymatic activity and for efficient escape from primary phagosomes (Bannam and Goldfine, 1999). The phospholipase activity of PLC-A can be detected on ALOA chromogenic media, on which *Listeria* expressing PLC-A appear surrounded by a green halo (AES Laboratoires).

Signaling Induced by PLC-A PLC-A mediates endothelial cell activation, characterized by the production of ceramide, activation of NF-κB,

and an increased expression of E-selectin, which stimulates PMN rolling and adhesion (Schwarzer et al., 1998). In endothelial cells and in neutrophils, PLC-A also enhances the phosphoinositide metabolism and the generation of lipid mediators induced by listeriolysin O but is not able to promote this effect by itself when expressed by *L. innocua* (Sibelius et al., 1996a; Sibelius et al., 1999; Goldfine et al., 2000). In macrophages, *L. monocytogenes* infection induces a biphasic activation of NF-κ B. The first transient phase is stimulated by bacterial binding or by purified LTAs. The second persistent phase appears when the bacteria reach the cytosol and correlates with the intracellular expression of PLC-A and PLC-B (Hauf et al., 1997; Goldfine et al., 2000). In addition, PLC-A, along with LLO and PLC-B, is in part responsible for the Ca^{2+} signaling produced by *L. monocytogenes* infection. Indeed, instead of the three calcium spikes produced upon bacterial infection, a ΔplcA mutant generates only a single spike, with no consequence on the efficacy of entry (Wadsworth and Goldfine, 1999).

Role of PLC-A in Infection and Virulence PLC-A has a minor individual role in escape from the primary phagosome but rather acts in synergy with listeriolysin O and PLC-B to fulfill this function, as shown by using simple or double mutants (Camilli et al., 1993; Marquis et al., 1995; Smith et al., 1995). It has no role in cell-to-cell spread, suggesting no role in escape from the double membrane vacuole (Smith et al., 1995). In macrophages, *L. innocua* expressing PLC-A are able to grow inside phagosomes but do not escape from these vacuoles, while *L. innocua* fail to replicate intracellularly, suggesting that PLC-A may possess a secondary function required during intracellular growth (Schwan et al., 1994). *plcA* mutants are slightly less virulent in mice after intravenous inoculation and are defective for liver but not spleen infection (Camilli et al., 1993). However, double ΔplcA-ΔplcB mutants are much less virulent than ΔplcB mutants, confirming the synergy of the two phospholipases in promoting an efficient infection (Smith et al., 1995).

PHOSPHOLIPASE PLC-B The phospholipase PLC-B is a 29-kDa protein with phosphatidylcholine phosphohydrolase activity (PC-PLC, also named lecithinase) produced by *L. monocytogenes* and *L. ivanovii* (Ralovich et al., 1972; Geoffroy et al., 1991; Vazquez-Boland et al., 1992). PLC-B is encoded by the gene *plcB* and is expressed as an inactive precursor. It can be activated by proteolytic cleavage involving the zinc-dependent metalloprotease Mpl, encoded by the gene *mpl*, and also by cellular proteases (Geoffroy et al.,

1991; Raveneau et al., 1992; Marquis et al., 1997). The *plcB* and *mpl* genes are part of an operon, which is controlled by the transcriptional activator PrfA (Mengaud et al., 1991b). The gene *plcB* is homologous to the genes encoding the lecithinases of *Bacillus cereus* and *Clostridium perfringens* (Vazquez-Boland et al., 1992).

Production and Activation of PLC-B In vitro, the PLC-B precursor and its activator Mpl are both secreted and tightly but noncovalently associated with the bacterial cell wall (Snyder and Marquis, 2003). During intracellular growth, translocation of PLC-B is inefficient and it remains at the membrane-cell wall interface. Upon acidification of phagosomes, pools of inactive PLC-B are translocated across the bacterial cell wall (Marquis et al., 1997; Snyder and Marquis, 2003). Activation of PLC-B requires the cleavage of a propeptide. The Mpl-dependent cleavage of PLC-B correlates with its cell wall translocation (Yeung et al., 2005). However, it was shown that PLC-B activation also occurs through a Mpl-independent process mediated by a lysosomal cysteine protease (Marquis et al., 1997).

Enzymatic Activities of PLC-B PLC-B is a zinc-dependent, calcium-independent enzyme, with an optimal activity at pH 5.5–7, in agreement with its role in acidic phagosomes containing *L. monocytogenes* (Geoffroy et al., 1991; Marquis and Hager, 2000). It has a lecithinase activity and is weakly hemolytic (Geoffroy et al., 1991). PLC-B has a broad substrate spectrum and hydrolyzes several lipids, including PC, phosphatidylethanolamine or phosphatidylserine and, to a lesser extent, sphingomyelin and PI (Geoffroy et al., 1991; Goldfine et al., 1993).

Signaling Induced by PLC-B Like PLC-A, PLC-B induces endothelial cell activation characterized by the production of ceramide, activation of NF-κ B, and an increased expression of E-selectin, which stimulates PMN rolling and adhesion, an event which might be important during systemic listeriosis (Schwarzer et al., 1998). Similarly, PLC-B mediates the persistent phase of NF-κ B activation in macrophages when delivered into the cytoplasm during *L. monocytogenes* infection (Hauf et al., 1997). In addition, PLC-B, along with LLO and PLC-B, mediates the Ca^{2+} signaling produced by *L. monocytogenes* infection, which is normally characterized by three calcium spikes. Instead, a Δ*plcB* mutant generates only the first spike resulting in a delayed bacterial internalization rate (Wadsworth and Goldfine, 1999).

Role of PLC-B in Infection and Virulence PLC-B deficient mutants, generated by deletion of the *plcB* or *mpl* gene, are less virulent in mice after intravenous injection (Raveneau et al., 1992). PLC-B also plays a crucial role during murine cerebral listeriosis, since Δ*plcB* mutants are strongly attenuated in the intracranial model of infection (Schluter et al., 1998). PLC-B is involved in the lysis of the double membrane vacuole and therefore in cell-to-cell spreading, as shown with a Δ*plcB* mutant, which forms small plaques on fibroblast cultures (Vazquez-Boland et al., 1992). In certain cell types, such as HenLe 407 and HeLa, but not in macrophages, PLC-B lyses the membrane of primary phagosomes containing *L. monocytogenes* in the absence of listeriolysin (Gaillard et al., 1987; Bielecki et al., 1990; Marquis et al., 1995; Grundling et al., 2003). This suggests that depending on the cell type, PLC-B may act alone or only help LLO to lyse the primary vacuole.

The Sphingomyelinase SmcL of Listeria ivanovii The sphingomyelinase SmcL is produced by the ruminant pathogen *L. ivanovii*. It is encoded by the gene *smcL*, which is present only in *L. ivanovii* but is highly homologous to the sphingomyelinase of *Staphylococcus aureus*, *Bacillus cereus* and *Leptospira interrogans*. SmcL expression is not controlled by the transcriptional regulator PrfA. Its enzymatic activity is responsible for the bizonal hemolysis of *L. ivanovii* and for the CAMP-like reaction, which corresponds to the synergistic hemolysis of *Rhodococcus equi* and *L. ivanovii* (Figs. 4 and 5). SmcL is required for virulence in mice and has a membrane damaging activity, which is required for vacuolar escape of *L. ivanovii* and its intracellular growth (Gonzalez-Zorn et al., 1999). The crystal structure of SmcL has been solved at a 1.9 angstroms resolution, revealing that SmcL adopts a DNasel-like fold and may bind to phospholipids (Openshaw et al., 2005).

ACTA ActA is the protein that mediates actin-based motility (Cossart and Bierne, 2001; Frischknecht and Way, 2001). ActA plays a key role in intracellular movements of *L. monocytogenes*, cell-to-cell spread, and consequently bacterial dissemination into host tissues (Kocks et al., 1992). ActA mutants grow as microcolonies in infected cells (Fig. 33) and their virulence is strongly affected (Domann et al., 1992; Brundage et al., 1993). ActA has also been reported to be required for *L. monocytogenes* entry into epithelial cells (Suarez et al., 2001) and for the recognition of the heparan sulfate receptor at the cell surface (Alvarez-Dominguez et al., 1997).

ActA is a surface protein, which is 610 amino acids long after cleavage of the signal peptide (Domann et al., 1992; Kocks et al., 1992). Note that articles differ in the way they number posi-
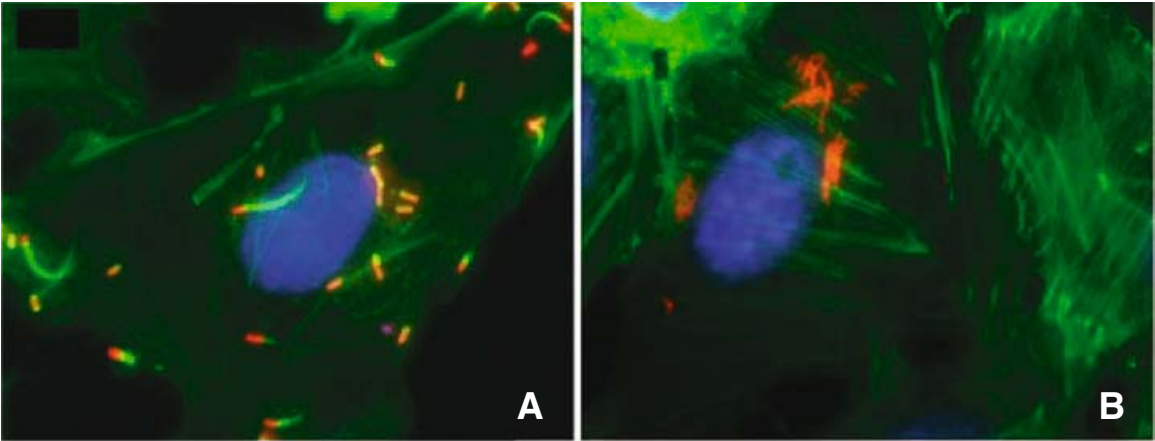
Fig. 33. Microcolonies formed by an ActA mutant in Vero cells. After infection for 6 h with *L. monocytogenes* wild-type (A) or ActA mutant (B), Vero cells were fixed. Actin was stained with rhodamine phalloidin (red) or with an anti-*L. monocytogenes* antibody. The ActA mutant, unable to move intracellularly, develops as microcolonies (arrows in B).

Fig. 34. ActA structure and critical regions. Schematic representation of ActA domain structure and sequence alignment with WASP (Wiskott-Aldrich syndrome protein). A, acidic domain; AB, actin-binding site; C, cofilin homology domain; MA, membrane anchor; P, PI-binding site; PRR, proline-rich repeats; PS, peptide signal. From Cossart and Bierne (2001), with permission.
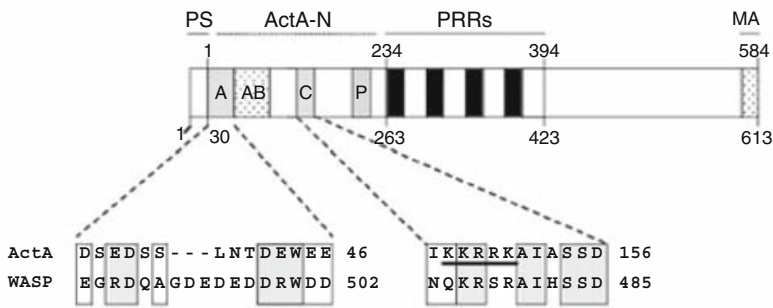
tions in the sequence, the codon 1 being either the first codon of the gene (Kocks et al., 1992) or that of the mature protein (Domann et al., 1992).

ActA is anchored to the bacterial membrane by its C-terminal hydrophobic region. Interestingly this region also allows anchoring of heterologous proteins on the mitochondrial surface when these proteins are expressed in eukaryotic cells (Pistor et al., 1994; Friederich et al., 1995). The ActA protein comprises a highly charged N-terminal domain (amino-acids 1–233), a central part made of four proline-rich repeats (amino acids 234–394) and a C-terminal part (amino acids 395–584) (Fig. 34). The ActA protein is a dimer, as originally shown by a yeast two-hybrid approach (Mourrain et al., 1997). In mammalian cells, it is phosphorylated, but the role of this phosphorylation, if any, has not been addressed (Brundage et al., 1993). ActA has been shown to bind phosphoinositides in vitro (Cicchetti et al., 1999; Steffen et al., 2000), but this property has not been investigated in detail. ActA could titrate PIP2, a phosphoinositide that controls the activity of some actin-binding proteins such as capping protein (Steffen et al., 2000).

ActA is polarly distributed on the bacterial surface (Fig. 35). Efforts to understand the origin of this polar distribution have led to the conclusion that the protein accumulates on the two old poles during growth and before division and is not incorporated at the septum during cell division (Kocks et al., 1993). After division, ActA is thus essentially located at one pole and absent from the new pole (Fig. 35). Interestingly, although the polar distribution of ActA, when discovered, appeared quite striking in view of its function in the formation of a polar actin tail (Smith et al., 1995), it has now been shown that many other, but not all, listerial surface proteins display a similar distribution. InlA, for example, is polarly distributed on the bacterial surface (Lebrun et al., 1996), but the amidase Ami does not seem to be polarly distributed (Braun et al., 1997).

ActA is necessary and sufficient to promote actin polymerization. This was first shown by expression of the *actA* gene after transfection in mammalian cells (Pistor et al., 1994). In this experiment, the ActA protein induced actin polymerization and aberrant membrane deformation when targeted to the inner face of the plasma membrane (Friederich et al., 1995). The critical role of ActA was demonstrated by expression of *actA* in the closely related species
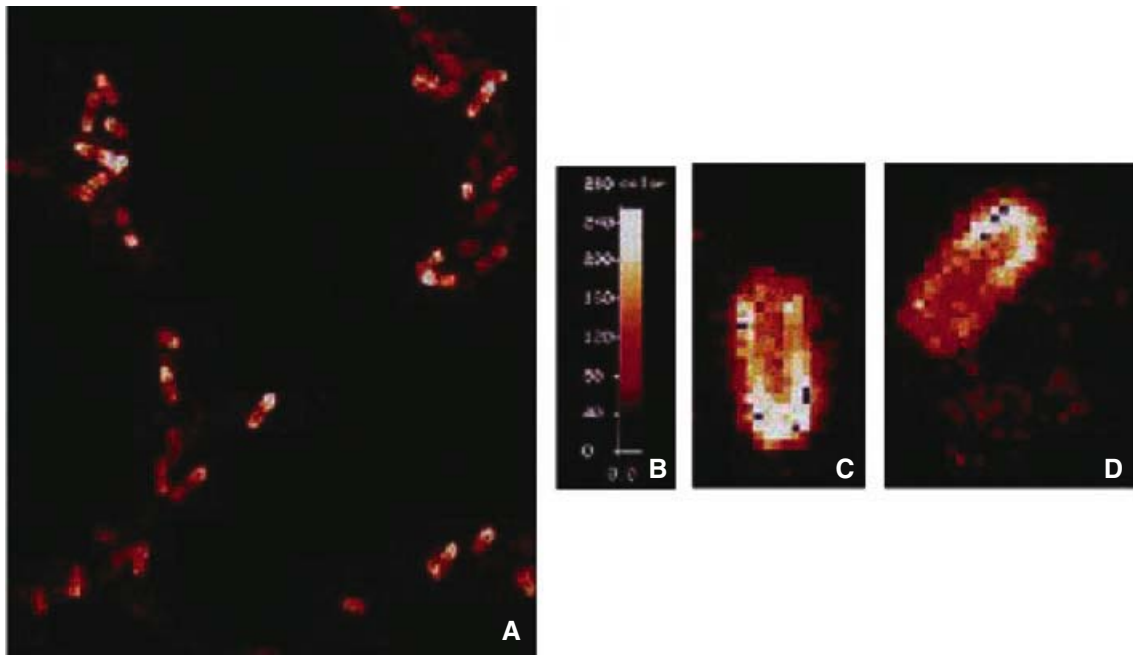
Fig. 35. Polarized distribution of ActA at the *L. monocytogenes* surface. Confocal microscopy images of *L. monocytogenes* labeled with ActA specific antibodies detected with fluorescent secondary antibodies large view (A) and close up (C and D). Linear scale of fluorescence intensities corresponding to images A, C and D (B). Scale bars, 10 μm (A) and 1 μm (C and D). From Kocks et al. (1993), with permission.

Listeria innocua, a bacterium that is totally non-pathogenic and lives in the environment. *Listeria innocua* expressing *actA* were able to move in cell extracts (Kocks et al., 1995). The final demonstration was achieved by the generation of latex beads coated with ActA, which, like *L. innocua* expressing ActA, were able to move inside cell extracts (Cameron et al., 1999).

Genetic analysis has greatly contributed to the analysis of the ActA protein and its mode of action. It was first shown that the N-terminal part of ActA is the critical part for movement. Bacteria expressing a chimera made of the N-terminal part of ActA and the ω fragment of LacZ can move in cell extracts, demonstrating that the first 233 amino acids are sufficient for movement (Lasa et al., 1995). A series of converging analyses, combined with biochemical approaches, has then demonstrated that this region is structurally and functionally similar to WASP/N-WASP (Wiskott-Aldrich syndrome protein) family proteins of eukaryotic cells and binds actin and Arp2/3 (Lasa et al., 1997; Skoble et al., 2000; Boujemaa-Paterski et al., 2001; Skoble et al., 2001; Auerbuch et al., 2003).

WASP/N-WASP proteins, first identified as mutated proteins in genetic disorders such as the Wiskott-Aldrich syndrome, communicate signals downstream from activated small GTPases to the actin cytoskeleton (Machesky and Insall, 1998). They interact with and activate the multiprotein complex Arp2/3, which is normally inactive in resting cells. Upon activation of cells, e.g. after interaction of a growth factor with its receptor, small GTPases can become activated—they switch from a GDP form to a GTP form—and are thus able to interact directly or indirectly with WASP/N-WASP family proteins, which in turn recruit and activate the Arp2/3 complex. This complex is a seven-protein complex able to stimulate de novo actin nucleation and the generation of a branched meshwork of actin filaments that grow from the sides of existing filaments leading to distinctive 70° branches. The C-terminal part of WASP/N-WASP proteins (called VCA or CA domain) stimulates the actin nucleating activity of Arp2/3 complex. This VCA domain is made of a N-terminal verprolin homology region, which binds actin (V or AB for actin binding in Fig. 34), a hydrophobic region termed the central region C, and a C-terminal segment rich in aspartate and glutamate termed the acidic region A in (Fig. 34). The primary Arp2/3 binding site is the A region, while the C region acting in concert with the V region and a bound actin monomer may drive the conformational changes necessary to stimulate nucleation (Machesky and Insall, 1998). Mutations in the 5′ end of the *actA* gene, in the regions encoding peptidic segments similar to the C and A regions, have definitively established similarities between ActA and other Arp2/3 activators. However, it is important to note the amino-acid stretches that bind Arp2/3 and actin in ActA, in contrast to

WASP/N-WASP family proteins, are not contiguous (Pistor et al., 2000; Skoble et al., 2000; Boujemaa-Paterski et al., 2001). Thus *L. monocytogenes* mimics activation of the Arp2/3 complex by WASP/N-WASP family proteins to achieve motility. However, Arp2/3 activation alone is not sufficient to promote actin-based motility in vitro (Loisel et al., 1999). An actin filament capping protein, either CapZ or gelsolin, and the filament depolymerizing and severing protein ADF/cofilin are also required to achieve motility. CapZ or gelsolin ensure that the actin polymerization driving *Listeria* motility is limited to uncapped filaments closely apposed to the bacterium, whereas ADF/cofilin ensures that actin monomers are made available by depolymerizing actin at the pointed end of the filaments in the tail (Loisel et al., 1999) (Fig. 36).

Another important component of the system, although not absolutely essential, is VASP whose absence decreases the rate of motility ten times (Geese et al., 2002). VASP binds to the proline rich region of ActA. VASP by its property to recruit profilin, which in turn facilitates the formation of ATP-actin from ADP-actin, could provide polymerization competent actin monomers to the ActA-Arp2/3 machinery (Grenklo et al., 2003). Other hypotheses have been proposed (Skoble et al., 2001; Bear et al., 2002; Auerbuch et al., 2003; Samarin et al., 2003). VASP by its ability to compete with capping proteins could stimulate filament elongation (Bear et al., 2002). Alternatively, VASP could increase branch spacing (Samarin et al., 2003).

While ActA is now recognized as a bacterial protein mimicking WASP/N-WASP family proteins, it is important to insist on the very instrumental role that this protein has played in deciphering the role of the Arp2/3 complex in actin-based motility. Indeed, while Arp2/3 complex had been isolated from a profilin-sepharose column in 1994 (Machesky et al., 1994), its role had remained totally mysterious until Mitchison and colleagues fractionated platelet cell extracts and showed that the fraction containing the Arp2/3 complex was able to stimulate an actin cloud around ActA-expressing *Listeria* and that ActA was able to activate Arp2/3 (Welch et al., 1997; Welch et al., 1998). The next important step was the two-hybrid approach used by Machesky and colleagues who showed that one of the subunits of the Arp2/3 complex (used as a bait) binds to WASP/N-WASP family proteins (Machesky and Insall, 1998). Then it was shown that WASP coated beads can move in cell extracts as do *Listeria* bacteria (Yarar et al., 1999). There is no better example of such a large contribution of a bacterial protein to the understanding of a key cell biology process, i.e., actin-based motility. How the force generated by the actin polymerization itself is producing movement is now being studied in detail with ActA-coated objects (Bernheim-Groswasser et al., 2002).

Several other bacteria can use actin to move intracellularly. *Listeria ivanovii* uses a protein similar to ActA (Gouin et al., 1995). Interestingly, *Shigella* uses the outer membrane protein IcsA that recruits N-WASP, which in turn recruits and activates Arp2/3 (Egile et al., 1999). In contrast, *Rickettsia* express an outer membrane protein, RickA, that is similar to WAVE, a member of the WASP/N-WASP family proteins and recruits the Arp2/3 complex (Gouin et al., 2004). However, the actin filaments in the *Rickettsia* tails are totally unbranched raising the possibility that another factor is unbranching the filaments or that the RickA protein itself is also unbranching the filaments. Possibly, other bacteria use variations on the theme to promote their intracellular movements. Recent reports
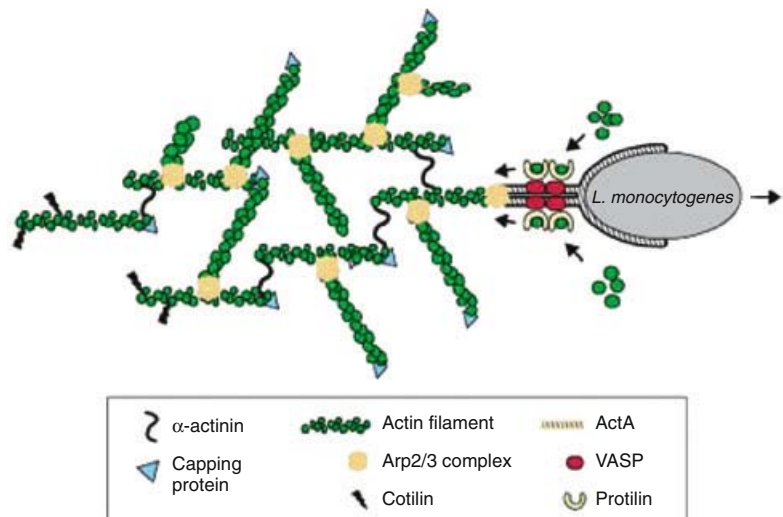
Fig. 36. Model of actin assembly induced by ActA. ActA is represented as a dimer localized in the hatched area in agreement with its polar distribution. The bacterium is moving from left to right (arrow). From Cossart and Bierne (2001), with permission.

indicate that *Mycobacterium marinum* (Stamm et al., 2003) and *Burkholderia pseudomallei* (Breitbach et al., 2003) are other examples of intracellular bacteria able to move inside cells.

PEPTIDOGLYCAN AND CELL WALL ASSOCIATED POLYMERS Peptidoglycan (PG) is one of the principal components of the cell wall of Gram-positive bacteria, along with associated polymers, such as the teichoic acids (TAs) and lipoteichoic acids (LTAs) (Fig. 37). It is a thick and rigid structure that covers the plasma membrane, protecting the cytoplasmic content (Merchante et al., 1995). The peptidoglycan and its associated polymers (TAs and LTAs) participate in the maintenance of the bacterial architecture and in anchoring bacterial surface proteins (Jonquieres et al., 1999; Navarre and Schneewind, 1999; Cossart and Jonquieres, 2000). They also possess several biological functions, such as resistance to lysozyme (Kamisango et al., 1982), bacteriophage receptors (Wendlinger et al., 1996), modulator of intracellular signaling in response to infection (Greenberg et al., 1996; Hauf et al., 1997), mitogenicity (Hether et al., 1983b; Paquet et al., 1986), and bacterial adhesion to and invasion of eukaryotic cells (Cowart et al., 1990; Autret et al., 2001).

Composition of the Peptidoglycan The peptidoglycan is a polymer composed of peptides and carbohydrates forming a dense and thick tridimensional network (20–50 nm). The glycans are organized as polymers of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues connected by a β1-4 bond (Fig. 38). The glycan heteropolymers are bound by peptidic bridges through the lactyl group of the MurNAc residue, allowing the constitution of a reticulate network (Archibald et al., 1993). The peptidic polymers are composed of a pentapeptide L-Ala-D-Glu-*meso*-diaminopimelic acid (*m*DAP)-D-Ala-D-Ala and are connected by a direct transpeptidation of the *m*DAP residue with the D-Ala residue of the peptidic bridges (Schleifer and Kandler, 1972; Fiedler et al., 1988; Archibald et al., 1993).

Synthesis and Degradation of the Peptidoglycan Despite its robustness, the peptidoglycan is not an inert structure and the rapid multiplication of *L. monocytogenes* suggests a rapid turnover for
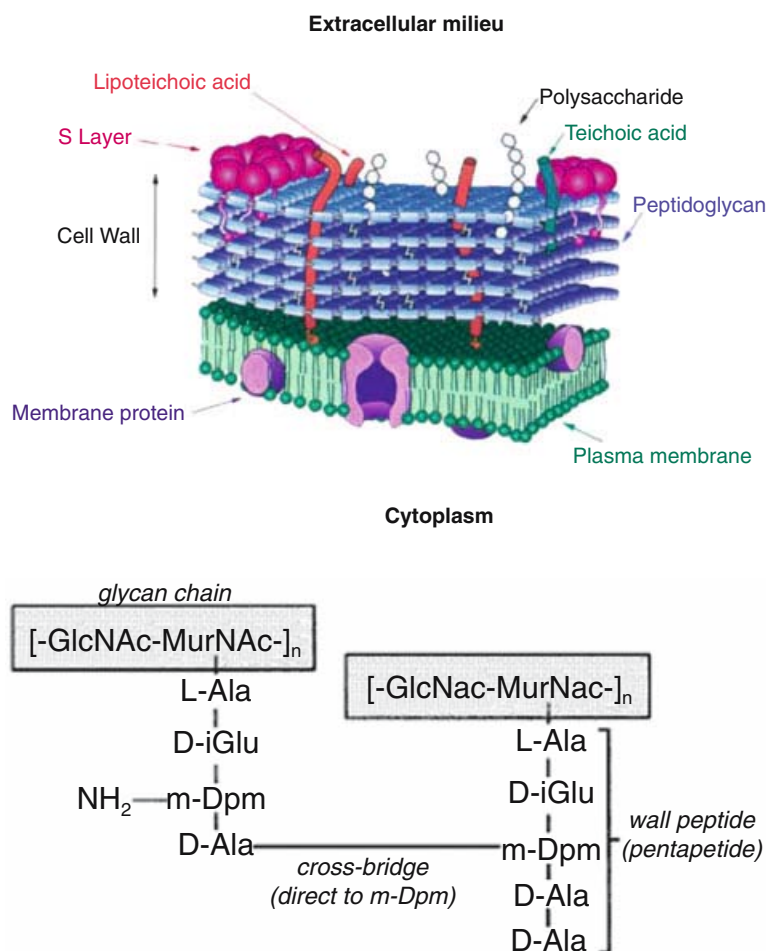
Fig. 37. Model representing a Gram-positive bacterial envelope and its major constituents. Note that a S-layer has not been described in the case of *L. monocytogenes*. From Delcour et al. (1999), with permission.

Fig. 38. Schematic representation of the structure of *L. monocytogenes* peptidoglycan. From Navarre and Schneewind (1999), with permission.

its cell wall components, including the peptidoglycan. The peptidoglycan synthesis occurs as a four-step process. First, there is a sequential ATP-dependent addition of the pentapeptide to the UDP-Mur*N*Ac residues. The newly formed molecule binds the lipid transporter undecaprenol-phosphate to the plasma membrane, which releases the UDP residue. The addition of Glc*N*Ac from the UDP-Glc*N*Ac forms lipid II, which is exported to the external face of the membrane. Finally, the lipid II transfers Glc*N*Ac-Mur*N*Ac-pentapeptide groups to the forming peptidoglycan. The transglycosylation and transpeptidation steps are made by diverse penicillin-binding proteins (Navarre and Schneewind, 1999). The rapid degradation of the peptidoglycan is mediated by autolysins and endolysins, produced by *L. monocytogenes* or its bacteriophages, respectively (Fig. 39). Autolysins and endolysins can cleave different bonds of the peptidoglycan, including the Mur*N*Ac(1-4)Glc*N*Ac (*N*-acetylmuramidases), the Glc*N*Ac(1-4)Mur*N*Ac (*N*-acetylglucosaminidases), the bond between the lactyl group of Mur*N*Ac and the amino group of L -Ala of the branched polypeptide (*N*-acetylmuramoyl-L -alanine amidases), the branched polypeptide at the bond between D-*iso*-Glu and the *m*DAP residues (endopeptidases), and the peptide bridges cross-linking the peptidoglycan between the L-Ala and D-Gln residues (L-alanoyl-D-glutamate peptidases) (Ghuysen et al., 1966; Schleifer and Kandler, 1972).

Composition of the Cell Wall Associated Polymers In *L. monocytogenes*, these polymers comprise TAs and LTAs. In other Gram-positive bacteria, they also include the teichuronic acids and the polysaccharides. These associated polymers are most of the time essential for bacterial growth and viability (Neuhaus and Baddiley, 2003).

TAs are electronegative polymers of ribitol-phosphates covalently bound to the peptidoglycan (Fig. 40). They are substituted by D -Ala residues and diverse sugars, which vary depending on serotypes (Ruhland and Fiedler, 1987; Fiedler et al., 1988; Fischer et al., 1988). LTAs are polymers of glycerophosphate substituted by Ala, galactose, and lipid residues (Fig. 41).

Fig. 39. Schematic representation of autolysin cleavage sites in *L. monocytogenes* peptidoglycan. From Lenz et al. (2003), with permission.

Fig. 40. Schematic representation of the structure of *L. monocytogenes* teichoic acids as well as the linking unit connecting them to the peptidoglycan. From Navarre and Schneewind (1999), with permission.

X = –H$_1$ –D-Ala$_1$ –**Galactose**

Recently, the *dlt* operon, which catalyzes the incorporation of D -Ala residues into LTAs, has been characterized. It comprises four genes, *dltA* to *dltD*, with *dltA* supposedly encoding the D-alanine-D-alanyl carrier protein ligase (Abachin et al., 2002). The glycerophosphate chain of LTAs is uncovalently anchored to the outer leaflet of the plasma membrane through a lipid anchor, composed of galactose bound to glycerol and substituted by fatty acids (Gal[α1-2]Glc[α 1-3]diacyl-glycerol) (Hether and Jackson, 1983a; Ruhland and Fiedler, 1987; Greenberg et al., 1996). Contrary to TA, the LTA structure does not vary with serotypes (Fiedler et al., 1988). However, in some *Listeria* strains, LTAs possess an additional phosphatidyl residue, which strengthens their association with the plasma membrane (Fischer et al., 1990). The LTAs are both membrane associated and secreted in the growth medium (Fischer et al., 1988). Their X-ray structure suggests that they adopt a compact organized micellar form with a length of 10–20 nm. Since the cell wall thickness ranges from 20 to 50 nm, some membrane LTAs are probably not exposed to the external medium (Labischinski et al., 1991).

Functions and Biological Activities of the Peptidoglycan and Associated Polymers

Functions Important for Bacterial Structure Probably the most important function of PG and its associated polymers, TAs and LTAs, resides in their participation in the maintenance of the cell wall and bacterial structures. Because of their negative charges, TAs are proposed to participate in the maintenance of a polarized membrane, as well as an electrochemical gradient within the cell wall, which locally concentrates certain ions (Hugues et al., 1973; Jolliffe et al., 1981; Kemper et al., 1993). Similarly, LTAs appear to favor the local concentration of Mg^{2+} ions, which may stabilize the plasma membrane (Roberts et al., 1985). LTAs regulate the activity of autolysins, which are involved in the peptidoglycan degradation required for bacterial growth and division (Fischer et al., 1981).

Role in Anchoring Bacterial Surface Proteins and as Bacteriophage Receptors PG and LTAs are also essential in that they anchor several *L. monocytogenes* surface proteins. Indeed, LPTXG-containing proteins, such as certain internalins, are covalently anchored to the PG allowing their exposure at the bacterial surface. This anchoring is mediated by SrtA (Navarre and Schneewind, 1999). Recently, another sortase, sortase B (SrtB), has been characterized in *L. monocytogenes*. It is required for the anchoring of a small subset of surface anchored proteins and probably recognizes its targets through a NXZTN sorting motif (Bierne et al., 2004). The gene encoding SrtB (*srtB*) is homologous to the *Staphylococcus aureus srtB* gene (Mazmanian et al., 2002). Both are part of an operon that contains at least one of their respective targets and exhibits a Fur (ferric uptake regulator) box, suggesting that their expression might be regulated by iron. One of the substrates of SrtB is the surface protein SvpA (Bierne et al., 2004), which was proposed to act as a virulence factor of *L. monocytogenes* (Borezee et al., 2001). By analogy with the SrtB of *S. aureus*, *L. monocytogenes* SrtB was proposed to be involved in bacterial adaptation to environmental conditions (Mazmanian et al., 2002; Bierne et al., 2004). InlB binds to the LTAs through its GW modules, although this noncovalent linkage appears to be relatively labile since InlB can also be released in the medium (Braun et al., 1998; Jonquieres et al., 1999). Ami, an autolysin involved in bacterial adhesion, also binds to the bacterial surface through its GW modules (Braun et al., 1997). It has also been suggested that the ribose groups of TAs and their associated cations may stabilize external bacterial proteins, protecting them from proteolysis (Chambert and Petit-Glatron, 1999; Hyyrylainen et al., 2000). Finally, the glycan residues of PG and TAs act as receptors for bacteriophages at the surface of *L. monocytogenes* (Wendlinger et al., 1996).

Role in Infection and Immunity In addition to its role in resistance to lysozyme (Kamisango et al., 1982), many reports point to a role of PG in stimulating the immune response. PG was shown early on to activate macrophages and to have

some mitogenic and adjuvant activities (Saiki et al., 1982; Hether et al., 1983b; Paquet et al., 1986). PG induces the production of the migration inhibition factor (Paquet et al., 1986). It enhances the NK activity in vivo and the generation of cell-mediated toxicity against tumor target cells (Saiki et al., 1982; Paquet et al., 1986). PGs were initially proposed to interact with the Toll-like receptor TLR2. However, the use of highly purified fraction of *L. monocytogenes* PGs revealed that they are detected intracellularly by the Nod2 protein and that LTAs contaminants contained in PGs preparations were sensed by TLR2 (Chamaillard et al., 2003; Girardin et al., 2003; Travassos et al., 2004).

TAs share some of the PG properties. They activate macrophages and are mitogenic (Hether et al., 1983b; Paquet et al., 1986). Interestingly, *L. monocytogenes* TAs appear to play a role in adhesion to and entry into epithelial cells. It was recently shown that the glycosylation of TAs favor these processes (Cowart et al., 1990; Autret et al., 2001).

LTAs bind to scavenger receptors on macrophages (Greenberg et al., 1996) and are sensed by TLR2 on epithelial cells (Travassos et al., 2004). In the *L. monocytogenes* strain LO28, a *dltA* mutant affected in the ability to incorporate D-Ala residues into LTAs is less virulent in the mouse model and less adherent to macrophages and epithelial cells (Abachin et al., 2002), suggesting a major role for peptidoglycan in virulence. However, deletion of the *dltA* gene in strain EGD seems to have less effect (Mandin et al., 2005).

AUTOLYSINS AND ENDOLYSINS *Listeria monocytogenes* produces several autolysins that digest its own cell wall peptidoglycan and are therefore listeriolytic. *Listeria monocytogenes* autolysins are proposed to be involved in numerous cellular processes including cell growth and division, septation, cell wall turnover, and peptidoglycan maturation, motility, protein secretion, and virulence (Ward and Williamson, 1984; Blackman et al., 1998; Smith et al., 2000). In addition, certain bacteriophages of *L. monocytogenes* produce endolysins, which are cell wall hydrolases synthesized during late gene expression of the lytic cycle. Endolysins enable the release of progeny virions from infected bacteria through degradation of the bacterial peptidoglycan (Loessner and Scherer, 1995a).

The profile of *L. monocytogenes* peptidoglycan hydrolases comprises multiple bacteriolytic enzymes, as determined by using renaturing SDS gel electrophoresis, with gels containing different bacterial cell wall extracts. Interestingly, the bacteriolytic profiles of different *Listeria* species appear very heterogeneous,
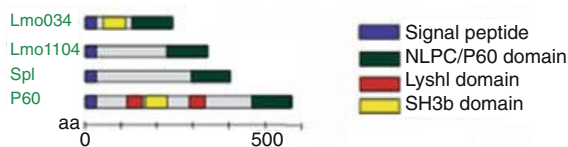
Fig. 42. Alignment of p60-like proteins. Determined from the annotation of the *L. monocytogenes* genome sequence. From Cabanes et al. (2002), with permission.

highlighting the specificity of these enzymes (MacLaughlan and Foster, 1997). Several *L. monocytogenes* autolysins have been more characterized, including p60, p45, Ami, NamA/MurA, and Auto (Wuenscher et al., 1993; Schubert et al., 2000; Carroll et al., 2003; Lenz et al., 2003; Cabanes et al., 2004). In addition, the *L. monocytogenes* genome sequence determination revealed seven other putative autolysins, with amidase or glucosamidase homology domains (Glaser et al., 2001; Cabanes et al., 2004) (Fig. 42).

Autolysin and Endolysin Activities The peptidoglycan of *L. monocytogenes* is composed of a copolymer of GlcNAc alternating with MurNAc, from which are branched polypeptide side chains. Crosslinks result from transpeptidation between the D-alanine of one peptide side chain and the free amino group of the *m*DAP of another (Dhar et al., 2000). Autolysins and endolysins are classified according to their hydrolytic specificity towards the peptidoglycan bonds (Fig. 39). *N*-acetylmuramidases and *N*-acetylglucosaminidases cleave the MurNAc(1-4)GlcNAc or the GlcNAc(1-4)MurNAc bonds, respectively. *N*-acetylmuramoyl-L-alanine amidases cleave the bond between the lactyl group of MurNAc and the amino group of L-Ala of the branched polypeptide. Endopeptidases directly cleave the branched polypeptide at the bond between D-*i*Glu (isoglutamine) and the *m*DAP residues (Ghuysen et al., 1966). L-Alanoyl-D-glutamate peptidases cleave the peptide bridges crosslinking the peptidoglycan between the L-Ala and D-Gln residues (Schleifer and Kandler, 1972).

Autolysins

p60 The autolysin p60 is encoded by the *iap* (invasion-associated protein) gene, which is transcribed independently of the transcriptional activator PrfA and whose expression is controlled at the post-transcriptional level (Kohler et al., 1991; Bubert et al., 1997; Bubert et al., 1999). It is both secreted and associated with the bacterial cell

wall (Kuhn and Goebel, 1989; Ruhland et al., 1993; Wuenscher et al., 1993). Secretion is mediated by the recently identified auxiliary secretion system SecA2, which mediates the secretion of at least seventeen secreted and surface proteins of *L. monocytogenes* (Lenz et al., 2003). p60 is expressed by all *Listeria* species with specific protein sequences for each species (Bubert et al., 1992b; Bubert et al., 1994; Gutekunst et al., 1992a). This finding can be exploited as a way to identify *Listeria* species (Bubert et al., 1992a; Bubert et al., 1994). On the basis of its similarity to LytF from *Bacillus subtilis*, p60 is proposed to have a D-*i*Glu-*m*DAP endopeptidase activity (Lenz et al., 2003).

In agreement with its peptidoglycan activity, p60 has been shown to be important for septum formation during bacterial division and therefore for bacterial viability (Wuenscher et al., 1993). For a long time, Δ*iap* mutants could not been obtained, suggesting that the protein was essential for bacterial viability. Therefore, the role of p60 was first evaluated in rough mutants expressing lower levels of p60 and forming long filamentous structures composed of bacterial chains (Kuhn and Goebel, 1989). The rough mutants are less virulent and enter less efficiently in certain eukaryotic cells, suggesting a role for p60 in bacterial invasion (Kuhn and Goebel, 1989; Gutekunst et al., 1992b; Hess et al., 1995). A viable Δ*iap* mutant was recently obtained, allowing more precise studies of the role of p60 in virulence. As for rough mutants, they also had a defect in septum formation and in virulence after intravenous infection of mice. In addition, the Δ*iap* mutant is impaired in bacterial movement and spreading from cell to cell because of an improper localization of the ActA at the surface *L. monocytogenes*. This abolishes the ability of ActA to polarly polymerize actin and to generate normal comet tails (Lenz et al., 2003; Pilgrim et al., 2003a).

p60 plays an important role in the immune response towards *L. monocytogenes* infection. Antibodies specific for p60 can act as opsonins and may play a role in preventing systemic infections in immunocompetent individuals (Kolb-Maurer et al., 2001). Moreover, p60 is a major protective antigen that induces both T-CD8 and Th1 protective immune responses, highlighting that both cellular and humoral immunity are important to fight *L. monocytogenes* infection (Harty and Pamer, 1995; Bouwer and Hinrichs, 1996; Geginat et al., 1998; Geginat et al., 1999).

p45 The autolysin p45, encoded by the *spl* gene, was identified using a monoclonal antibody raised in mice against heat-killed bacteria. It is a 45-kDa protein with peptidoglycan lytic activity against *L. monocytogenes*. p45 is homologous to the autolysin p60 of *L. monocytogenes* and to Gram-positive secreted proteins of unknown activity, P54 of *Enterococcus faecium*, and Usp45 of *Lactococcus lactis*. p45-like proteins are present in all *Listeria* species, except *L. grayi*. p45 is found both as a secreted protein, as expected from the presence of a 27-amino acid signal peptide, and associated with the cell wall, in a strong but noncovalent fashion, as determined by SDS extraction. This behavior is reminiscent of p60 and of the two main effectors of *L. monocytogenes* invasion, InlA and InlB (Schubert et al., 2000).

Ami The autolysin Ami is a 102-kDa protein produced by *L. monocytogenes*. It is probably an amidase according to the sequence homology of its catalytic domain with other amidase domains (Braun et al., 1997). It is associated to the bacterial surface by a domain containing a series of GW modules, similar to those of one of the major proteins required for *L. monocytogenes* invasion, InlB (Braun et al., 1997; Jonquieres et al., 1999) (Fig. 19B). There is increasing evidence that certain autolysins, like Ami, may act as complementary adhesins during infection. Indeed, inactivation of Ami in mutants devoid of either one or two of the main effectors of *L. monocytogenes* invasion, InlA and/or InlB, resulted in a strong reduction of adhesion to hepatocytes, HepG2, and to the enterocyte-like cell line, Caco-2. On the contrary, inactivation of Ami alone affected only slightly the adhesion to hepatocytes, HepG2 (Milohanic et al., 2001). As for InlB, the cell wall-anchoring (CWA) domain containing the GW modules appears to promote Ami adhesion to cells (Heilmann et al., 1997; Hell et al., 1998; Milohanic et al., 2001). As a probable consequence of its role in adhesion, a mutant with inactivated Ami is slightly attenuated in the liver of mice infected intravenously, indicating a role for Ami in *L. monocytogenes* virulence (Milohanic et al., 2001). Ami from two different epidemic serovars (1/2a and 4b) were compared and their CWA domains were shown to be variable, as compared with the rest of the molecule. The purified CWA domain of Ami from serovar 4b binds less efficiently to Hep-G2 cells as compared with that of serovar 1/2a (Milohanic et al., 2004).

NamA/MurA Two recent concomitant studies identified a new peptidoglycan-hydrolyzing enzyme in *L. monocytogenes*, which was named by the authors "NamA" for *N*-acetylmuramidase or "MurA" for muramidase (Carroll et al., 2003; Lenz et al., 2003). Comparative analysis of biochemical and genetic properties of the two molecules revealed NamA is the same protein as MurA (D. Cabanes, personal communication).

Characterization of the auxiliary protein secretion system, SecA2, revealed that the secretion of several proteins was dependent on this system, including the autolysin p60 and a protein homologous to NamA (Lenz et al., 2003). In parallel, a cell wall hydrolase (MurA) encoded by the *murA* gene was detected by a *L. monocytogenes*-specific monoclonal antibody (EM-7G1), which also recognizes the autolysin p60 (Carroll et al., 2003).

MurA is a major cell surface protein of *L. monocytogenes*. The C-terminal domain of MurA contains four copies of a KM repeat motif, which is also present in p60 and is proposed to be involved in cell wall anchoring. MurA shares homologies with the autolysin p60 and with muramidases. In agreement with this, MurA exhibits a peptidoglycan lytic activity specific for *Micrococcus lysodeikticus*. A deletion mutant of *mur*A lacking the cell wall hydrolase activity is affected in *L. monocytogenes* autolysis and grows as long chains during exponential growth, as a consequence of a septation defect (Carroll et al., 2003). The role of MurA in cell infection and in virulence was not tested in this study, but the study of NamA revealed a moderate role in *L. monocytogenes* persistence in mouse organs (Lenz et al., 2003).

Auto Recently, a novel autolysin encoding gene, *aut*, was identified. It is the only autolysin gene that is absent from the nonpathogenic bacteria *L. innocua*. The *aut* gene is expressed independently of the virulence gene regulator PrfA and encodes a surface protein, Auto, with an autolytic activity, as expected from the presence of a domain harboring homologies with autolysin encoding genes, especially *N*-acetylglucosaminidases. The protein Auto possesses a C-terminal cell wall-anchoring domain made up of four GW modules, similar to those observed in the other autolysin Ami and in InlB, one of the major invasion proteins of *L. monocytogenes*. The morphology of a Δ*aut* deletion mutant was similar to those of the wild-type, with no defect in septation and cell division, suggesting no role for Auto in these functions (Cabanes et al., 2004).

Auto is required for entry of *L. monocytogenes* into different nonphagocytic eukaryotic cell lines but is not required for efficient adhesion, formation of comet tails, or cell-to-cell spreading. A Δ*aut* deletion mutant has a reduced virulence following intravenous inoculation of mice and oral infection of guinea pigs, which correlates with its low invasiveness. However, the autolytic activity of Auto by itself, rather than an invasive ability, might be critical for virulence. Indeed, Auto may control the general surface architecture exposed to the host by *L. monocytogenes* and/or the composition of the surface

products released by the bacteria, highlighting the possible direct role of autolysins in pathogenicity (Cabanes et al., 2004).

FlaA Listeria monocytogenes can move by means of flagella-based motility. The flagellum is composed of a single protein, FlaA (Dons et al., 1992). FlaA also facilitates initial contact with epithelial cells and contributes to effective invasion in vitro (Dons et al., 2004). It was shown that the purified FlaA protein functions as a peptidoglycan hydrolase (Popowska and Markiewicz, 2004). The flagellar protein FlgJ of *Salmonella*, was previously shown to have peptidoglycan hydrolyzing activity, locally digesting the murein sacculus to permit assembly of the rod structure of the flagellum (Nambu et al., 1999). FlaA of *L. monocytogenes* is the first gram-positive flagellar protein demonstrated to have a peptidoglycan hydrolyzing activity.

Endolysins Three endolysins, Ply118, Ply500 and Ply511, produced respectively by the bacteriophages A118, A500 and A511, have been characterized in *L. monocytogenes*. They induce rapid lysis of *Listeria* strains from all species but not of other bacteria (Loessner and Scherer, 1995a). Ply511 is an *N*-acetylmuramoyl-L-alanine amidase, while Ply118 and Ply500 are L-alanoyl-D-glutamate peptidases (Schleifer and Kandler, 1972). A cell wall binding site has been characterized in the C-terminal domains of Ply118 and Ply500 as being sufficient to direct the enzymes to their substrates (Loessner et al., 2002). *Listeria innocua* carries a cryptic phage 2438, which produces the Cpl2438 enzyme highly similar to Ply500 (Zink et al., 1995). A gene homologous to the Ply118 endolysin was detected in the genome sequence of the cryptic phage of *L. monocytogenes* strain EGDe (Glaser et al., 2001). The specificity of these endolysins for *Listeria* cells was exploited in different applications, such as rapid in vitro lysis of *Listeria* cells (Loessner et al., 1995b; Dhar et al., 2000), or programmed self-destruction of intracellular *Listeria* cells within the cytosol of macrophages (Dietrich et al., 1998).

OTHER FACTORS INVOLVED IN *L. MONOCYTOGENES* INFECTION Several factors have been involved in the *L. monocytogenes* infection process either at the level of adhesion, entry, escape from the phagosome, or intracellular multiplication, or at an unknown step.

LAP LAP (*Listeria* adhesion protein) is a 104-kDa protein present both in the cytoplasm and secreted by *L. monocytogenes*. LAP is expressed by all *Listeria* spp. except by *L. grayi* (Pandiripally et al., 1999). LAP mediates *L.*

monocytogenes binding to intestinal epithelial cells but not to nonintestinal epithelial cells, such as liver, kidney or skin cells. Interestingly, the specificity of LAP is even more restricted since it is required for full adhesion to intestinal epithelial cells lines from the ileum-cecum and colon but not from the duodenum or jejunum. These results suggest that LAP may play a role during the intestinal phase of the infection (Jaradat et al., 2003). The heat shock protein Hsp60 has been reported to act as a cellular receptor for LAP on the intestinal cell line Caco-2 (Wampler et al., 2004).

SvpA SvpA (surface virulence-associated protein) is a 64-kDa protein both anchored at the bacterial surface by SrtB and secreted in the extracellular medium (Borezee et al., 2001; Bierne et al., 2004). Its expression is not controlled by PrfA, the transcriptional activator of most virulence factors. A mutant deficient in SvpA was reported to be less virulent in the mouse model after intravenous infection (Borezee et al., 2001). However, the relevance of this result is unclear since virulence of a mutant deficient in SrtB, in which SvpA is no longer exposed at the surface, is not affected (Bierne et al., 2004). The growth defect of the *svpA* mutant may explain its virulence attenuation (H. Bierne and P. Cossart, unpublished results). Another possibility is that the expression of SvpA at the bacterial surface is not important for its role in virulence.

LpeA LpeA (lipoprotein promoting entry) is a 35-kDa protein identified by in silico analysis of the *L. monocytogenes* genome sequence. Despite its homology with PsaA, a *Streptococcus pneumoniae* adhesin, LpeA is not involved in adhesion of *L. monocytogenes*. Instead, it is required for entry into the intestinal and hepatic cell lines. Interestingly, a LpeA-deficient mutant survive longer in macrophages than wild-type bacteria and is slightly more virulent for mice (Reglier-Poupet et al., 2003b).

FbpA FbpA (fibronectin binding protein) is a 60-kDa protein identified by signature-tagged mutagenesis. It is required for efficient liver colonization of mice inoculated intravenously and for intestinal and liver colonization after oral infection of transgenic mice expressing human E-cadherin. FbpA binds to immobilized human fibronectin and increases adherence of wild-type *L. monocytogenes* to HEp-2 cells in the presence of exogenous fibronectin. Despite the lack of conventional secretion/anchoring signals, FbpA is detected on the bacterial surface. Strikingly, FbpA expression affects the secretion of two virulence factors, LLO and InlB, and coimmuno-precipitates with these two proteins. Thus, FbpA, in addition to being a fibronectin-binding protein, may behave as a chaperone or an escort protein for two important virulence factors and appears to be a novel multifunctional virulence factor of *L. monocytogenes* (Dramsi et al., 2004).

Stp Analysis of the *L. monocytogenes* EGDe genome sequence revealed the presence of putative eukaryotic-like phosphatases. Accordingly, the *stp* gene encodes a membrane associated Mn^{2+}-dependent serine-threonine phosphatase, required for *L. monocytogenes* virulence. Using a phosphoproteomic approach, the translation elongation factor EF-Tu was identified as the first target of Stp (Archambaud et al., 2004).

Vip Vip is a novel virulence factor of *L. monocytogenes*, identified by comparative genomics. Vip is a LPXTG surface protein anchored to the cell wall by sortase A. It is positively regulated by PrfA, the transcriptional activator of the major *L. monocytogenes* virulence factors. Vip is implicated in entry into some mammalian cells. Gp96, an endoplasmic reticulum resident chaperone protein (Li et al., 2002), which is also localized at the cell surface, was identified as a cellular receptor for Vip (Cabanes et al., 2005). The Vip-Gp96 interaction is critical for bacterial entry into cells. In murine models, Vip plays a role in *L. monocytogenes* virulence at the intestinal level and at late stages of the infectious process. Vip appears as a new virulence factor exploiting Gp96 as a receptor for cell invasion and/or signaling events that may interfere with the host immune response in the course of *Listeria* infection (Cabanes et al., 2005).

RESISTANCE TO STRESS *Listeria monocytogenes* is ubiquitous in nature and can infect many animal species. The pathogenic bacterium is adapted to survive and/or multiply under a wide variety of harsh environmental conditions, outside as well as inside the host. In food-processing plants, food products, and nature, *L. monocytogenes* has to contend with suboptimal growth conditions, such as refrigeration temperatures, heat, high osmolarity, high pH, and low water activity. Survival has been reported from −0.4°C to 45°C (Farber and Peterkin, 1991a), at salt concentrations up to 10% (McClure et al., 1991), alkaline conditions up to pH 9 (Cheroutre-Vialette et al., 1998). In the human host, *L. monocytogenes* encounters stressors such as lysozyme in saliva, blood, neutrophils and monocytes, reactive oxygen and nitrogen species in the host cell phagosomes, bile salts, digestive enzymes in the small intestine, and stressful microenvironments (such as nutrient deprivation in the host cell cytoplasm, acidic

pH of the stomach, and low oxygen tension and high osmolarity in the intestine). *Listeria monocytogenes* survives and/or grows at a pH as low as 4, even 3 after acid adaptation (Cotter and Hill, 2003) and in the presence of a high concentration of bile (Begley et al., 2002). *Listeria monocytogenes* has evolved a series of adaptive responses to cope with this large variety of stresses.

The first step in stress survival strategies is the sensing of stress conditions and/or stressors and the transduction of a signal leading to an appropriate response (Fig. 43). Two-component signal transduction systems are used for stress sensing by many bacteria, including *L. monocytogenes*. Deletion of *L. monocytogenes* LO28 *lisK*, the gene encoding the membrane-associated sensor histidine kinase of the LisRK two-component system, affects acid and ethanol tolerance and attenuates virulence in mice (Cotter et al., 1999). Using a deletion mutant of the response regulator gene *lisR*, Kallipolitis et al. confirmed the role of the LisRK system in virulence, acid and ethanol tolerance. LisR was also shown to be involved in response to oxidative stress, as growth of the *lisR* mutant was strongly inhibited by 0.025% hydrogen peroxide. Two other response regulators contribute to pathogenicity and stress response in *L. monocytogenes* LO28 strain, a regulator showing 52% identity with the potassium uptake protein KdpE of *Clostridium acetobutylicum* and a regulator showing 43% identity with the *Enterococcus faecium* VanR protein involved in vancomycin resistance (Kallipolitis and Ingmer, 2001).

Many stress response mediators have been identified in *L. monocytogenes*, including chaperones that maintain protein integrity under damaging conditions and proteases that degrade damaged proteins. The DnaK heat shock chaperone is required for survival of *L. monocytogenes* under high temperatures and acidic conditions, as well as for phagocytosis by macrophages (Hanawa et al., 1995; Hanawa et al., 1999). The major heat shock chaperones GroES and GroEL are induced at high temperature (Hanawa et al., 1995; Gahan et al., 2001), at low pH and during infection of J774 cells (Gahan et al., 2001). The ClpC stress protein, an ATPase that belongs to the HSP-100/Clp family, is required for survival under iron deprivation, high temperature or high osmolarity, in bone marrow macrophages and in organs of mice infected by *L. monocytogenes* (Rouquette et al., 1996; Rouquette et al., 1998). Two other proteases have been reported to be involved in stress response and virulence, namely the HSP-100 family member ClpE (Nair et al., 1999) and the serine protease ClpP (Gaillot et al., 2000). Several other proteins involved in specific stress responses have been characterized in *L. monocytogenes*. The major cold shock protein of *L. monocytogenes* is the nonheme ferritin-like protein Flp, also designated Fri (Hebraud and Guzzo, 2000). Fri promotes adaptation to nutritional and thermal shifts, provides protection against reactive oxygen species *in vitro* and is required for full virulence of *L. monocytogenes* (Olsen et al., 2005; Dussurget et al., 2005). The glutamate decarboxylase system plays an important role in survival at low pH found in food (Cotter et al., 2001b) and in gastric fluid (Cotter et al., 2001a). The F_0F_1-ATPase could also contribute to acid tolerance of *L. monocytogenes*
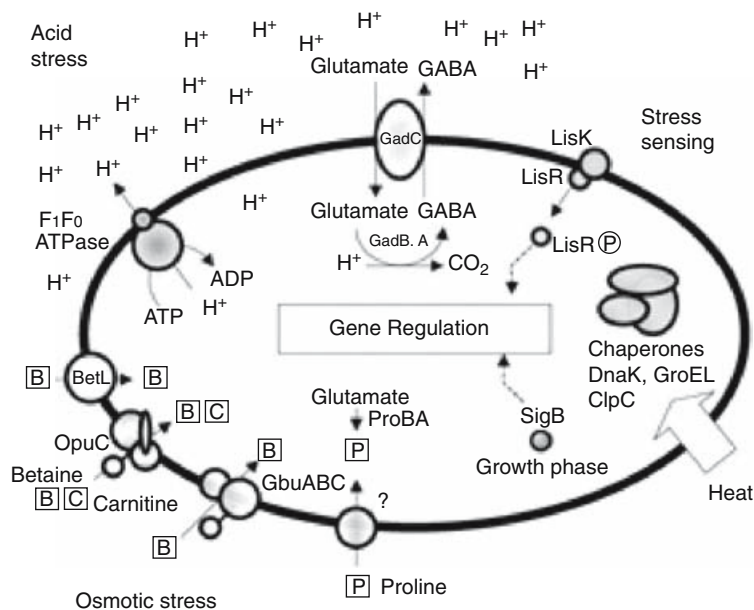
Fig. 43. Schematic representation of the stress resistance process in *L. monocytogenes*. From Hill et al. (2002), with permission.

(Cotter et al., 2000). Adaptation to osmotic stress depends on the intracellular accumulation of osmolytes, e.g., betaine, carnitine and proline (Ko et al., 1994; Fraser et al., 2000). Uptake systems include the Na$^+$-dependent betaine porter I BetL (Sleator et al., 1999), the ATP-dependent betaine porter II GbuABC (Ko and Smith, 1999), the carnitine uptake systems OpuC (Fraser et al., 2000; Sleator et al., 2001a; Sleator et al., 2003; Wemekamp-Kamphuis et al., 2002), and the oligopeptide permease OppA (Borezee et al., 2000). In addition, one osmolyte synthesis system has been described in *L. monocytogenes*, that of proline. Mutation of the *proAB* operon leads to an increased sensitivity to salt (Sleator et al., 2001a). Several of these systems have been shown to play a role in the virulence potential of *L. monocytogenes*, e.g., OpuC (Sleator et al., 2001b) and OppA (Borezee et al., 2000). Bile tolerance of *L. monocytogenes* involves the bile salt hydrolase Bsh, an enzyme that deconjugates bile salts and that is required for both intestinal and hepatic phases of listeriosis (Dussurget et al., 2002; Begley et al., 2005). The transporter BtlA (Begley et al., 2003) and other systems (such as the putative transporter of the glutamate decarboxylase GadE, the penicillin V amidase Pva, the bile tolerance protein BtlB, the bile exclusion system BilE and the zinc uptake regulator ZurR) contribute to tolerance to bile or various other stresses, e.g., low pH, salt, ethanol, detergents and antibiotics (Begley et al., 2002; Sleator et al., 2005a; Begley et al., 2005).

Stress-responsive sigma factors play an important role in regulating expression of both virulence genes and stress response genes in bacteria, including *L. monocytogenes*. The alternative sigma factor sigma B contributes to the ability of *L. monocytogenes* to survive and/or multiply under stressful conditions outside the host, e.g., acid, osmotic or oxidative stresses (Becker et al., 1998; Ferreira et al., 2001; Ferreira et al., 2003), low temperature (Becker et al., 2000) or carbon starvation (Ferreira et al., 2001). Sigma B also plays a role in the capacity of *L. monocytogenes* to persist within the host during the infectious process. It has been demonstrated that sigma B contributes to transcription of the virulence gene activator PrfA (Nadon et al., 2002). Characterization of the sigma B-dependent general stress regulon confirmed the broad role of this sigma factor. Indeed, the regulon includes genes encoding both general stress response proteins (e.g., the RNA-binding protein Hfq, glutamate decarboxylase GadB, general stress protein Ctc, and the glutathione reductase), and virulence factors (e.g., the internalins InlA and InlB, the bile salt hydrolase Bsh, the bile exclusion system BilA and the stress-responsive solute transporter OpuC (Sleator et al., 2001b; Kazmierczak et al.,

2003; Sue et al., 2003; Christiansen et al., 2004; Kim et al., 2004; Sleator et al., 2005a). *Listeria monocytogenes* stress response is also controlled by CtsR, a transcriptional repressor of *clpC*, *clpE* and *clpP*. A *ctsR* deletion mutant was not affected for virulence in mice and displayed an increased survival at high temperature and under salt stress (Nair et al., 2000). Finally, the regulatory RNA-binding protein Hfq is required for resistance to osmotic and ethanol stress, and full virulence in mice (Christiansen et al., 2004).

Gene Regulation in *Listeria*

Analysis of the complete genome sequence of *L. monocytogenes* EGDe revealed its extraordinary regulatory capacity as 201 regulatory proteins have been identified (Glaser et al., 2001). The *L. monocytogenes* genome contains the highest proportion of regulatory genes (7%) after that of *Pseudomonas aeruginosa* (8.4%) (Stover et al., 2000). This observation is in line with the fact that *L. monocytogenes* is an ubiquitous, opportunistic pathogen that needs a variety of combinatorial pathways to adapt its metabolism to a given niche. However, only a few of these regulators have been studied experimentally.

TWO-COMPONENT SYSTEMS The *L. monocytogenes* genome contains several two-component systems consisting of 15 histidine kinases and 16 response regulators. Several of them have been studied in detail: *lisR/lisK* (Cotter et al., 1999; Cotter et al., 2002), *cheY/cheA* (Flanary et al., 1999), *agrA/agrC* (Autret et al., 2003), *cesR/cesK* (Kallipolitis et al., 2003) and *virS/virR* (Mandin et al., 2005). The LisR/LisK system is involved in acid, ethanol and oxidative stress, and the inactivation of *lisRK* resulted in a slight decrease of virulence (Cotter et al., 1999). The LisRK signal transduction system is also involved in the sensitivity of *L. monocytogenes* to nisin and cephalosporins (Cotter et al., 2002). The genes regulated by this system are a putative penicillin-binding protein (*lmo2229*), a histidine kinase (*lmo1021*), and a protein of unknown function (*lmo2487*). A novel role for the two-component regulatory system LisRK in osmosensing and osmoregulation has been shown (Sleator and Hill, 2005b). Furthermore, *htrA*, a gene linked to osmotolerance and virulence potential in *L. monocytogenes*, was reported to be under the transcriptional control of LisRK (Stack et al., 2005).

Insertional inactivation of the *L. monocytogenes cheYA* operon abolished response to oxygen gradients and reduced flagellin expression and affected the ability of *L. monocytogenes* to attach to the mouse fibroblast cell line 3T3 (Flanary et al., 1999). A deletion mutant *cheA*

had impaired swarming and the *cheY* and *cheYA* double mutants were unable to swarm on soft agar plates, suggesting that *cheY* and *chaA* genes encode proteins involved in chemotaxis (Dons et al., 2004). Autret and colleagues identified by signature-tagged mutagenesis the *agrABCD* locus (Autret et al., 2003). The production of several secreted proteins was modified, indicating that the *agr* locus influenced protein secretion. Although the ability of the mutant to invade and multiply in cells in vitro was not affected, virulence of the *agrA* mutant was affected in the mouse model, indicating that the *agr* locus is involved in virulence of *L. monocytogenes* (Autret et al., 2003).

Kallipolitis and colleagues inactivated five putative response regulators identified by using degenerate primers in *L. monocytogenes* strain LO28 (Kallipolitis et al., 2003). These correspond to the genes *lmo2583*, *lmo2678/kdpE*, *lmo2501/phoP*, *lmo2422/cesR*, and *lmo1377/lisR*. Three of these putative response regulators, *lmo2678/kdpE*, *lmo2422/cesR*, and *lmo1377/lisR*, contributed to pathogenicity in a mouse infection model either by intragastric or intraperitoneal injection. Recently, *cesRK* was studied in more detail (Kallipolitis et al., 2003), showing that this two-component system responds to the presence of cell wall-acting antibiotics and affects ß-lactam resistance.

The new two-component system of *L. monocytogenes*, named VirS/VirR, which is necessary for virulence, was recently identified using signature-tagged mutagenesis (Mandin et al., 2005). A transcriptomic analysis revealed that VirS controls the expression of 108 other genes, probably by crosstalking with another response regulator, and that 12 genes are regulated by VirR, including the *dlt* operon (Mandin et al., 2004), previously shown to be important for *L. monocytogenes* virulence (Abachin et al., 2002). A conserved DNA sequence located upstream of all the transcriptional units regulated by VirR was identified by in silico analysis, probably representing the DNA binding site of VirR. The role of all sixteen putative two-component systems of *L. monocytogenes* was undertaken systematically, by the introduction of in frame deletions into 15 out of the 16 response regulator genes and the resulting mutants were characterized. With one exception, the deletion of the individual response regulator genes has only minor effects on *in vitro* and *in vivo* growth of the bacteria. The mutant carrying a deletion in the orthologue of the *Bacillus subtilis* response regulator gene *degU* showed reduced virulence in mice, indicating that DegU is involved in the regulation of virulence-associated genes (Williams et al., 2005). Knudsen and colleagues have shown that the DegU response regulator is a

pleiotropic regulator involved in expression of both motility at low temperature and in vivo virulence in mice (Knudsen et al., 2004).

PrfA—Key Regulator of *Listeria monocytogenes* Virulence Genes The PrfA (positive regulatory factor A) protein of *L. monocytogenes* functions as a master regulator, which is required and directly involved in the control of the differential expression of the *L. monocytogenes* virulence genes. PrfA is a 237-amino acid protein encoded by the first gene of the virulence gene cluster of *L. monocytogenes* whose expression is under the control of this transcriptional regulator (Leimeister-Wachter et al., 1990; Mengaud et al., 1991b; Chakraborty et al., 1992; Dramsi et al., 1993). The *prfA* gene lies downstream from and is co-transcribed with *plcA-prfA* is transcribed as either a monocistronic or a bicistronic *plcA-prfA* transcript, thereby creating an autoregulatory loop essential for the appropriate expression of virulence genes (Mengaud et al., 1991b) (Fig. 44). Evidence for an overexpression of PrfA inside cells has been observed (Klarsfeld et al., 1994; Renzoni et al., 1999).

Molecular and Structural Features On the basis of structural and functional features shared with the cAMP receptor protein (Crp) of *E. coli*, PrfA is clearly a member of the Crp/Fnr (fumarate
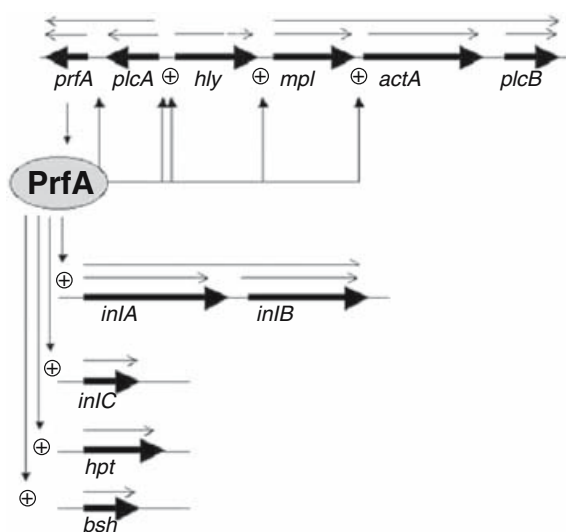
Fig. 44. The PrfA regulon in *L. monocytogenes*. *prfA*: positive regulatory factor; *plcA*: phosphatidylinositol phospholipase C; *hly*: listeriolysin O; *mpl*: metalloprotease; *actA*: actin-polymerization protein; *plcB*: broad-range phospholipase C (lecithinase); *inlA*, *inlB*: large cell wall associated internalins A and B; *inlC*: small secreted internalin, *hpt*: hexose phosphate transporter; *bsh*: bile salt hydrolase; and + indicates transcriptional induction. Thin arrows above the gene arrows indicate the different transcripts.

nitrate reductase regulator) family of transcriptional regulators (Lampidis et al., 1994). Interestingly this family of regulators seems to be of particular importance for *Listeria* insofar as there are 15 Crp/Fnr family members in *L. monocytogenes* in contrast to only one in *B. subtilis* and two in *E. coli* (Glaser et al., 2001). Figure 45 shows the domain organization of PrfA from *L. monocytogenes* compared to that of Crp from *E. coli*. In the N-terminal domain, both contain several ß-sheets delimited by glycine residues forming a ß-roll structure and an α-helical region. In Crp, both are involved in binding of the cofactor cAMP. In PrfA several of the amino acids required for cAMP binding are not conserved and the role of this ß-roll structure is not known yet. Two of the three activating regions of Crp, which mediate the interaction with the RNA polymerase, are conserved in PrfA (Fig. 45). PrfA has in its C-terminal region a DNA-binding helix-turn-helix (HTH) region with 70% similarity to the corresponding HTH in Crp. The crystal structure of PrfA has recently been solved confirming the above information (Thirumuruhan et al., 2003; Protein Data Bank (http://www.rcsb.org/pdb), accession code 1OMI; Eiting et al., 2005). Activation of genes by PrfA requires binding of this HTH region to a 14-bp palindromic sequence present at PrfA regulated promoters, the PrfA-box (Freitag et al., 1993; Bockmann et al., 1996; Sheehan et al., 1996). This palindrome is centered at position –41 relative to the transcriptional start site and partially over-laps the –35 promoter regions. It was shown that the two critical elements of PrfA-dependent promoters, the PrfA-box and the –10 box, can be functionally exchanged as long as the distance in between is maintained to 22 or 23 bp (Luo et al., 2005). PrfA activates all genes of the virulence gene cluster (*prfA*, *plcA*, *hly*, *mpl*, *actA* and *plcB*) as well as the *inlA* and *inlB* (Dramsi et al., 1997), *inlC* (Engelbrecht et al., 1996; Lingnau et al., 1996), *bsh* (Dussurget et al., 2002) and *hpt* (*uhpT*) genes (Chico-Calero et al., 2002). Table 6 shows the PrfA-box sequences of these PrfA regulated virulence genes in *L. monocytogenes*. That the PrfA box is indeed the recognition sequence for PrfA has been suggested by the effect of mutations in this sequence (Freitag et al., 1992). Further evidence came from gel retardation assays and in particular from DNAse I protection experiments (Bockmann et al., 1996). At the C-terminus, PrfA has an extension as compared to Crp with the characteristics of a leucine zipper (Lampidis et al., 1994). However, different studies indicate that the mechanisms regulating the *L. monocytogenes* virulence genes are very complex and show that PrfA is not the only regulatory factor. By using an *in vitro* transcription assay for *L. monocytogenes* genes, it was shown that overlapping PrfA-dependent and -independent promoters that are differentially activated by GTP are present for the PrfA-dependent genes *inlC* and *mpl*, indicating that these genes are not solely regulated by PrfA. Furthermore, PrfA-independent transcription of
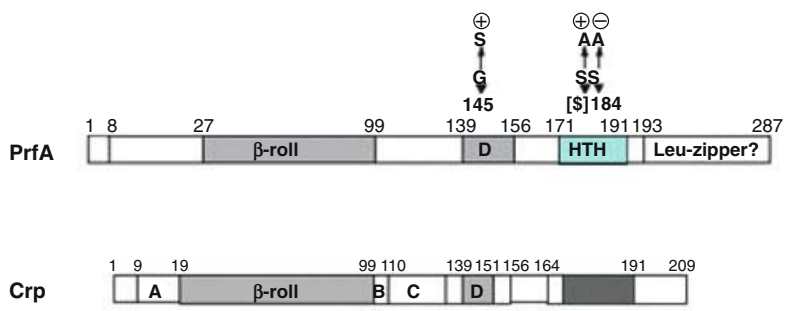
Fig. 45. Domain organization of PrfA (positive regulatory factor) from *L. monocytogenes* and Crp (cyclic AMP receptor protein) from *E. coli*. Numbers indicate start and end of domains. A-D: alpha helices in Crp, β-roll structure: cAMP binding region in Crp from *E. coli* and similar structure in PrfA. Leu-zipper?: putative leucine zipper. Adapted from Goebel et al. (2000), with permission.

Table 6. PrfA (positive regulatory factor)-box of PrfA regulated virulence genes in *L. monocytogenes*.

Promoter		PrfA-box	Spacer −10 box Startcodon
P2*hly* 5′	CA	TTAACATTTGTTAA	−N23-TAGAAT-N139-ATG-3′
P*plcA* 5′	CG	TTAACAAATGTTAA	−N22-TAAGAT-N31-TTG-3′
P*mpl* 5′	AA	TTAACAAATGTAAA	−N22-TATAAT-N156-ATG-3′
P*actA* 5′	GA	TTAACAAATGTTAG	−N21-GATATT-N157-GTG-3′
P*inlA* 5′	GG	ATAACATAAGTTAA	−N22-TATTAT-N402-GTG-3′
P*inlC* 5′	AT	TTAACGCTTGTTAA	−N22-TAACAT-N106-TTG-3′
P*hpt* 5′	TG	ATAACAAGTGTTAA	−N23-TATATT-N152-ATG-3′
P*bsh* 5′	AT	TTAAAAATTTTTAA	−N30-TATGAG-N109-ATG-3′
P*prfA* 5′	AG	CTAACAATTGTTGT	−N21-TATTTT-N37-ATG-3′
		TTAACANNTGTTAA	

inlC and *mpl* was shown to be strongly inhibited by PrfA because of the close proximity of the PrfA binding site to the −35 box (Luo et al., 2004). Shen and Higgins demonstrate that the *hly* 5′ UTR plays a critical role in regulating expression of LLO during intracellular infection (Shen and Higgins, 2005). They suggest that the *hly* 5′ UTR functions independently of PrfA-mediated transcription and can enhance expression of cis-associated genes through a mechanism that appears to act at both a post-transcriptional and post-translational level. Deletion of the hly 5′ UTR, while retaining the hly ribosome binding site, had a moderate effect on LLO production during growth in broth culture, yet resulted in a marked decrease in LLO levels during intracellular infection (Shen and Higgins, 2005).

Functional Changes Due to Alterations in Defined Positions   Specific amino acid changes in PrfA correlates with high or low level of hemolytic activity, because of high- or low-level expression of the *hly* gene. A Gly145Ser substitution (Fig. 45) is responsible for a high hemolytic phenotype (Ripio et al., 1997b). This mutation freezes PrfA in its active conformation leading to the expression of a constitutively active PrfA* form (Ripio et al.,1996). The crystal structure of PrfA* has been solved and compared to that of wild-type PrfA, showing that the HTH motifs and adopt a conformation similar to cAMP-induced Cap, which favors DNA binding (Eiting et al., 2005). The correlation of the high hemolytic phenotype with the altered PrfA protein is also supported by the observation that the transfer of the *prfA** gene to a strain with low hemolytic activity shifts it to a strain with high hemolytic activity (Bohne et al., 1996; Ripio et al., 1997b). A Leu140Phe mutation and an Ile45Ser mutation, mapping to the N-terminal β-roll structure, also cause a constitutive overexpression of the PrfA regulon (Vega et al., 2004; Wong and Freitag, 2004). Wong and Freitag report that the mutation Leu140Phe results in the aggregation of *L. monocytogenes* in broth culture and, unlike previously described *prfA* mutations, appears to be slightly toxic to the bacteria (Wong and Freitag, 2004). As shown by Sheehan and colleagues, a Ser183Ala exchange also leads to increased binding of PrfA causing enhanced expression of virulence genes, whereas a Ser184Ala exchange leads to decreased binding of PrfA to its target sequence and thus to reduced expression of virulence genes (Sheehan et al., 1996) (Fig. 45). Shetron-Rama and colleagues selected *L. monocytogenes* mutants expressing high levels of *actA* during *in vitro* growth after chemical mutagenesis (Shetron-Rama et al., 2003). This led to the identification

of two different amino acid substitutions within PrfA (Glu77Lys and Gly155Ser), which also appear to lock PrfA in its activated state. Both *prfA* Glu77Lys and *prfA* Gly155Ser strains are more efficient than wild-type bacteria in gaining access to the host cell cytosol and in initiating the polymerization of host cell actin, and both are capable of mediating LLO-independent lysis of host cell vacuoles in cell lines for which *L. monocytogenes* vacuole disruption normally requires LLO activity (Mueller and Freitag, 2005). Positive selection of mutations leading to loss or reduction of transcriptional activity of PrfA led to the identification of mutations in three regions of the PrfA protein: 1) between amino acids 58–67 in the β-roll domain, 2) between amino acid 169–193, corresponding to the DNA-binding HTH motif, and 3) in the 38 C-terminal amino acids of PrfA, which form the putative leucine-zipper-like structure. Mutations in the HTH motif and the leucine-zipper-like structure lead to PrfA proteins unable to bind to the PrfA-binding site (Herler et al., 2001).

Physicochemical Parameters Influencing PrfA A number of physicochemical parameters are known to affect the expression of PrfA-dependent genes. For example, elevated iron concentrations in the medium repress transcription of *hly* and *actA*, whereas growth in activated charcoal-containing BHI or in minimum essential medium results in induction of *prfA* and PrfA-dependent gene expression. Furthermore, the presence and utilization of different carbohydrates has a remarkable impact on the virulence of *L. monocytogenes* (Kreft and Vazquez-Boland, 2001). For instance, growth on glucose-1-phosphate (G-1-P) as sole carbon source is strictly PrfA-dependent (Ripio et al., 1997a). It was shown that the PrfA-dependent utilization of this compound is necessary for efficient cytosolic replication of *L. monocytogenes* (Chico-Calero et al., 2002). An interesting observation is that metabolizable unphosphorylated sugars inhibit the expression of PrfA-dependent virulence genes. This mechanism was first discovered with cellobiose, but later any fermentable carbohydrate was found to trigger the downregulation of PrfA-dependent virulence genes. CcpA (catabolite control protein A) was suggested to be an important element of carbon source regulation in *L. monocytogenes*, but utilizable sugars still downregulate the expression of *hly*. In a *ccpA* mutant, CcpA does not seem to be involved in carbon source regulation of virulence genes (Behari and Youngman, 1998). Interestingly, in the presence of cellobiose, PrfA is fully expressed, suggesting that PrfA is post-transcriptionally modified (Renzoni et al., 1997)

and can switch between a transcriptionally active and inactive form upon interaction with an hypothetical activating factor (Renzoni et al., 1997; Ripio et al., 1997b, Vega et al., 1998). This is also suggested by the fact that *L. monocytogenes* strains producing a constitutively active PrfA form are refractory to sugar-mediated virulence gene expression (Ripio et al., 1997a). Isolation of mutants that exhibit high levels of PrfA-controlled gene expression in the presence of cellobiose or glucose led to the identification of mutations in two different genetic loci, *gcr* and *csr*, both unlinked to the major virulence cluster. A mutation in *gcr* deregulates the expression of PrfA-controlled genes in the presence of several repressing sugars and other environmental conditions, a phenotype similar to that of a Gly145Ser substitution in PrfA itself. A mutation in the *csr* locus, within *csrA*, results in a cellobiose-specific defect in virulence gene regulation. Mutations in both *gcr* and *csr* are required for full relief of cellobiose-mediated repression of the PrfA regulon, suggesting the existence of two semi-independent pathways for cellobiose-mediated repression (Milenbachs Lukowiak et al., 2004). Expression of *prfA* and *hly* in murine macrophage-like J774.1 cells, with or without activation by IFN-γ plus LPS, showed that expression of *hly* inside activated macrophage was abolished by addition of SOD and catalase, suggesting that reactive oxygen intermediates contribute to the upregulation of *prfA* and *hly* transcriptions. Moreover, exposure of *L. monocytogenes* to H_2O_2 dramatically enhanced the expression of both *prfA* and *hly* mRNAs, suggesting that oxygen radicals generated in activated macrophages provide a positive signal for up-regulation *prfA* and the *prfA*-regulated virulence genes in *L. monocytogenes* (Makino et al., 2005).

The PrfA-regulon Analyzed at the Genome Level The PrfA-regulon and the influence of physicochemical factors on gene regulation by PrfA have been studied at the complete genome level by a transcriptomics approach (Milohanic et al., 2003; Rauch et al., 2005). Using whole-genome arrays based on the sequenced *L. monocytogenes* EGDe strain (Glaser et al., 2001), the expression profiles of two *L. monocytogenes* wild-type strains (EGDe and P14*) were compared to those of their *prfA*-deleted mutant strains. The study of three different growth conditions, rich-medium (BHI), BHI supplemented with active charcoal, and BHI supplemented with cellobiose allowed three groups of genes differently regulated by PrfA to be identified. One group included the already known virulence genes and two newly identified genes (*lmo2219*

and *lmo0788*), which all contain a PrfA-box. These results indicate that the core set of genes directly regulated by PrfA is quite small (i.e., in the conditions tested, only 12 genes [*hly*, *mpl*, *actA*, *plcB*, *plcA*, *prfA*, *inlA*, *inlB*, *inlC*, *hpt*, *lmo2219*, and *lmo0788*]). Most interestingly, a second group of genes encoding a putative sugar transport system was shown to be negatively regulated by PrfA, indicating that PrfA can also act as a repressor. The most surprising result was the identification of a third group of 53 genes (group 3), which was regulated but did not contain a PrfA binding site. In depth analysis revealed sigma B like promoters in front of nearly all the genes of this group 3, suggesting interplay between PrfA and sigma B (Milohanic et al., 2003). This finding is substantiated by a report by Nadon and colleagues who reported the P_2prfA is a sigma B dependent promoter (Nadon et al., 2002). A transcriptome analysis comparing a wild-type and a *sigB* deleted mutant strain using focused microarrays containing 208 putative sigma B regulated genes and known *L. monocytogenes* virulence genes showed that sigma B regulates the stress response. It also confirmed the previous implication of sigma B in regulation of virulence functions (Kazmierczak et al., 2003).

Temperature Regulation of PrfA Expression Pathogenic Listeriae face a sudden increase in temperature during the transition from the environment to a warm-blooded host. This temperature shift correlates with a maximal expression of the virulence genes at 37°C but nearly no expression at 30°C. The low expression of virulence genes coincides with the absence of PrfA protein, although the *prfA* gene is still transcribed (Leimeister-Wachter et al., 1992; Renzoni et al., 1997; Johansson et al., 2002). At low temperatures, *prfA* is transcribed from its own promoter, giving rise to a monocistronic *prfA* transcript. At 37°C, transcription originates both from the *prfA* promoter and from the PrfA-dependent *plcA*-promoter (Fig. 46). However, the absence of PrfA protein at temperatures lower than 30°C is not due to a general untranslatability of the *prfA* monocistronic messenger. The mechanisms underlying thermoregulated production of PrfA have recently been elucidated. At low temperature, the untranslated mRNA region (UTR) preceding *prfA* forms a secondary structure, which masks the ribosome-binding site. At high temperature, the structure is destabilized (Fig. 47). Thus, a RNA "thermosensor" controls the expression of virulence genes in *L. monocytogenes* (Johansson et al., 2002).

Regulation through Sigma Factors L. monocytogenes is predicted to contain only five sigma factors in comparison to 18 in *B. subtilis* (Kunst
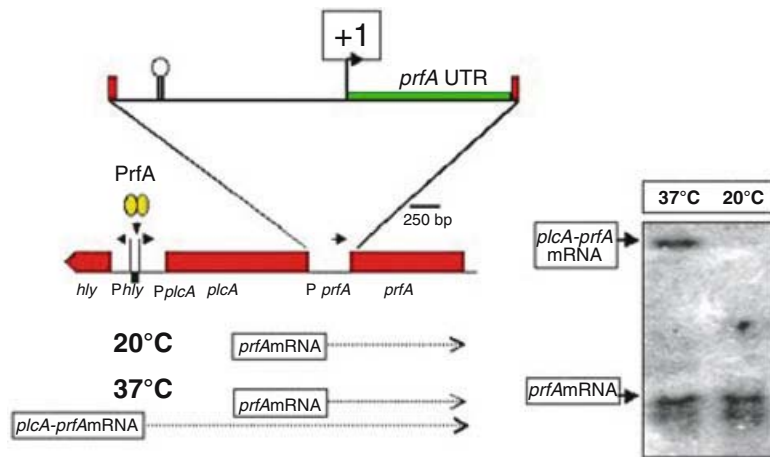
Fig. 46. Transcription of the *prfA* (positive regulatory factor) gene at both 20°C and 37°C. A) Schematic representation of *prfA* transcription. The mono- and the bicistronic mRNA at 20°C and 37°C are indicated as dotted flashes under the map. The intergenic region between *plcA* (phosphatidylinositol phospholipase C) and *prfA* are enlarged above the map indicating the transcriptional start (+1) and the *prfA*-UTR (untranslated region). B) Northern blot analysis of *prfA* transcription. Wild-type *L. monocytogenes* strain LO28 was grown to late logarithmic phase in brain–heart infusion (BHI) media at either 20° or 37°C. From Johansson et al. (2002), with permission.

Fig. 47. Model of mechanism underlying thermoregulated expression of PrfA (positive regulatory factor). The *prfA*-UTR (untranslated region) forms a secondary structure at low temperatures (30°C) masking the ribosomal region of *prfA*, thus preventing the binding of the ribosome. PrfA is not translated and virulence genes are not expressed. At high temperatures (37°C) the *prfA*-UTR partially melts, and thereby permits binding of the ribosome to the Shine-Dalgarno sequence. From Johansson et al. (2002), with permission.
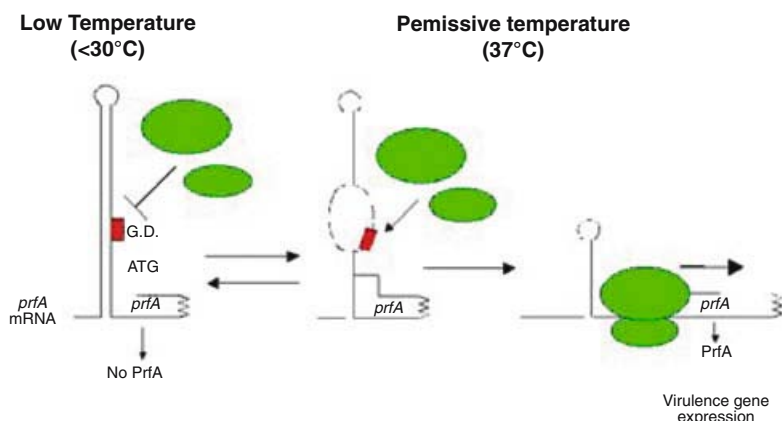
et al., 1997) and 13 in *Mycobacterium tuberculosis* (Cole et al., 1998). These are similar to *B. subtilis* SigA, SigB, SigH, SigL (RpoN, sigma 54) and ECF-type sigma factors. Sigma 54 of *L. monocytogenes* has been described to be involved in sensitivity to antibacterial peptides, the subclass IIa bacteriocins (Robichon et al., 1997). One sigma 54-dependent operon, *mptACD*, has been characterized in details in *L. monocytogenes*. Its expression is controlled by the ManR activator, which belongs to the LevR family (Dalet et al., 2001). The *mptACD* operon encodes the AB, C and D subunits of a PTS permease of the mannose family, EIItMan. The lack of *mptACD* expression, in a *mpt* or a *rpoN* mutant, leads to resistance of *L. monocytogenes* to subclass IIa bacteriocins. The EIItMan permease was thus proposed to be the receptor for these antibacterial peptides (Dalet et al., 2001; Duché et al., 2002; Gravesen et al., 2002). Study

of the role of the alternative sigma 54 factor by comparing the global gene expression and the protein content of *L. monocytogenes* strain EGDe suggested that sigma 54 is mainly involved in the control of carbohydrate metabolism in *L. monocytogenes* via direct regulation of the PTS activity, alteration of the pyruvate pool and modulation of carbon catabolite regulation (Arous et al., 2004).

The alternative sigma factor sigma B modulates the stress response of several Gram-positive bacteria, including *Bacillus subtilis* and the food-borne human pathogens *Bacillus cereus*, *L. monocytogenes* and *Staphylococcus aureus*. In all these bacteria, sigma B is responsible for the transcription of genes that can confer protection to the cell against adverse conditions. Upon exposure to stress, the stress has to be sensed and signaled through a regulatory cascade, leading to the activation of sigma B and, subsequently, to

the transcription of the set of sigma B-regulated genes (the Sigma B regulon). The encoded proteins perform specific functions, which protect the cell against stress. In *L. monocytogenes*, sigma B was shown to have a role in growth and survival under low temperatures, acid tolerance, survival under environmental, energy, and intracellular stress conditions (Becker et al., 2000; Wiedmann et al., 1998; Chaturongakul and Boor, 2004). This condition is of special interest for practical reasons, as chilled storage is often a crucial factor in the preservation of minimally processed foods. The deletion of *sigB* had only very minor effects on virulence, as tested in animals models (Wiedmann et al., 1998), even though several virulence factors in this organism are under the control of sigma B (see below). The *bsh* gene encoding the bile salt hydrolase and two genes from the internalin family, which contribute to bacterial entry are partially sigma B-dependent and also contribute to PrfA-mediated virulence in *Listeria monocytogenes* (Kazmierczak et al., 2003; Sue et al., 2003; Nadon et al., 2002; Milohanic et al., 2003). Furthermore, the central role of sigma B in the regulatory network is illustrated by the fact that sigma B can control other important regulatory proteins like the RNA-binding protein Hfq in *L. monocytogenes*. Hfq is thought to play a crucial role in the post-transcriptional regulation of gene expression by small RNAs and the dependency of this regulator on sigma B adds another level of complexity to the function of sigma B in *L. monocytogenes* (Christiansen et al., 2004).

Animal Models

Considerations for the Choice of an Adequate Animal Model An infection is a multistep process integrating a number of host and microbial variables. For this reason, comprehension of the pathophysiology of a human infection necessitates an animal model in which the infectious agent has the same cell and tissue tropism as in humans. Similarly, an animal model should allow observation of the same direct effects and indirect immunopathological damages occurring in humans. Such a model should allow testing the in vivo relevance of results acquired using more reductionist in vitro approaches. Ideally, both the microbial pathogen and the animal model should be genetically amenable, thus allowing assessment of the role of critical microbial and host factors during the infectious process. The genetic manipulation of both the pathogen and the host may lead to a better understanding of their respective contributions in the complex interplay that results in disease (Finlay, 1999; Harvill and Miller, 2000; Lecuit and Cossart, 2002).

Since animal listeriosis exists, animal species naturally infected by *L. monocytogenes* can be used as models to study the pathophysiology of human listeriosis. However, this also implies important technical limitations: the animals developing a disease closely resembling human listeriosis are not classical laboratory animals such as the rat or the mouse but rather farm animals such as sheep, cattle and goats. As for other human diseases, the murine model became the most widely used animal model in mammal biology. Indeed, mice have all the necessary qualities to make them good laboratory animals: smallness, resistance and adaptation to captivity, easy reproduction, abundant offspring, short gestation period, and a physiology and pathophysiology in most respects comparable to humans. However, certain human pathogens are not mouse pathogens, and mouse disease induced by human pathogens can be manifestly different from the actual human disease. With this in mind, the mouse is frequently regarded as an unsatisfactory model for the study of human infectious diseases, which still continues to be used in the absence of better models (Lecuit and Cossart, 2002).

A review of the literature did not find reports of symptomatic natural *L. monocytogenes* infection in mice and rats. Experimentally, the mouse, like the rat, which is phylogenetically extremely close to the mouse species, cannot be easily infected with *L. monocytogenes* by the oral route. Most studies using mice or rats fail to induce a reproducible lethal infection after oral infection with *L. monocytogenes*, even using extremely high inocula (Zachar and Savage, 1979; Roll and Czuprynski, 1990; Okamoto et al., 1994; Gaillard et al., 1996; MacDonald and Carter, 1996; Marco et al., 1997; Pron et al., 1998; Huleatt et al., 2001; Manohar et al., 2001). This implies that one has to consider with caution results of in vivo studies using dose-response mathematical models to determine the theoretical lethal dose in humans after oral ingestion of contaminated food (Haas and Thayyar-Madabusi, 1999).

To circumvent the low susceptibility of mice infected via the oral route, alternate routes of administration were used, although they do not mimic the natural route for infection. They include intravenous and intraperitoneal, or more rarely intranasal, subcutaneous, conjunctival, intracardiac or intracerebral routes, not only in mice but also in guinea pigs, rabbits and sheep (Kautter et al., 1963; Racz et al., 1970; Khan et al., 1972; Dustoor et al., 1977; Scheld et al., 1979; Prats et al., 1992). In mice, inoculation via the intravenous rather than the oral route can produce a lethal infection, and thus allows the determination of the LD_{50} and comparison of the virulence of various mutants. This route of

administration was particularly instrumental in characterization of most *L monocytogenes* virulence genes (Gaillard et al., 1986; Kathariou et al., 1987; Cossart et al., 1989b; Domann et al., 1992; Kocks et al., 1992; Raveneau et al., 1992). It also allowed demonstrating that immunosuppression, young age and gravidity were associated with a reduced LD_{50}. Finally, it revealed granulomatous hepatitis due to intravenously-acquired murine listeriosis was similar to that observed in *granulomatosis infantiseptica* (Luft and Remington, 1982; Stelma et al., 1987; Genovese et al., 1999). However, this symptomatic septicemic infection seems different from what is observed in the human species, for which the phase preceding the feto-placental or CNS infection is mostly clinically silent.

Infection by the intravenous route in the mouse model also allowed the discovery of cellular immunity and its cellular basis (CD8+ lymphocytes) (Mackaness, 1962; Mackaness and Hill, 1969; North, 1969; Pearson and Osebold, 1973; Kaufmann, 1988). The pioneering studies carried out by Mackaness in the 1960s made *L. monocytogenes* one of the best-characterized and most instrumental models of intracellular microbes (Mackaness, 1962; Mackaness and Hill, 1969). However, the low capacity of *L. monocytogenes* to infect mice via the oral route has limited the use of *L. monocytogenes* to the field of systemic immunity at the expense of mucosal immunity. This model of infection also made it possible to demonstrate that genetic background played a part in the susceptibility to *L. monocytogenes*, since BALB/c mice are more sensitive to *L. monocytogenes* than C57 BL/6 following intravenous inoculation (Cheers and McKenzie, 1978). The molecular mechanisms underlying these susceptibility differences are unknown. Their identification should lead to a better understanding of the human inter-individual variation in susceptibility to *L. monocytogenes* (Boyartchuk et al., 2001).

ADDRESSING THE SPECIES-SPECIFICITY OF *L. MONOCYTOGENES* IN ANIMAL MODELS A number of human pathogens exhibit stringent host specificity. For some of them, the molecular basis of their restricted host-tropism has been at least partially deciphered. It implicates the species-specific interaction of a microbial ligand with its cellular receptor(s), as demonstrated for viruses such as the poliovirus, the measles virus, HIV and hepatitis C virus, but also for bacteria such as *Neisseria gonorrhea* and *Neisseria meningitidis*, and more recently *L. monocytogenes* (Dalgleish et al., 1984; Mendelsohn et al., 1989; Dorig et al., 1993; Alkhatib et al., 1996; Dragic et al., 1996; Virji et al., 1996; Chen et al., 1997; Gray-Owen et al., 1997; Pileri et al., 1998; Lecuit et al.,

1999; Lecuit et al., 2001b). Study of a species-specific ligand-receptor interaction, thought to play a critical role in human infection, should be possible in an animal model that allows this interaction to occur. Two possibilities exist to establish such a model: the simplest one is to identify an animal species in which the interaction does occur (such as cattle, sheep and goats for *L. monocytogenes*) and to use it as a model. A more sophisticated approach is to generate a genetically modified animal, generally a transgenic mouse line that expresses the human receptor and to look at the effect of the transgene expression on the infectious process. The human receptor can be expressed either in place of or in addition to its nonfunctional mouse orthologue, either ubiquitously or under the control of a tissue-specific promoter that drives expression of the transgene in the cell types targeted by the pathogen during the human infection (Lecuit and Cossart, 2002).

As mentioned above, in mice, oral inoculation is a very inefficient way to trigger systemic listeriosis, because *L. monocytogenes* translocation across the intestinal barrier is low and not higher than that of the closely related nonpathogenic species *L. innocua*. The few detectable foci of bacterial multiplication are restricted to Peyer's patches, areas that contain M-cells, i.e., cells possessing a phagocytic activity. Thus, *L. monocytogenes* is not an enteropathogen for mice. Moreover, in mice, the brainstem and the feto-placental unit do not appear as elective targets, as is the case in humans (M. Lecuit, unpublished results).

Following mouse intravenous inoculation, LLO, ActA, PLC-A, and PLC-B but not internalin act as virulence factors. Indeed, despite the well-established prominent role of internalin in the internalization process in vitro, its role in vivo has long remained elusive since an internalin mutant behaves as its wild-type parent after mouse intravenous or oral inoculation. Strikingly, shortly after the discovery of human E-cadherin as the internalin receptor, it was established that, in contrast to human E-cadherin, mouse E-cadherin is unable to promote entry of *L. monocytogenes* into cells. This specificity was shown to depend on a single amino acid of the mature E-cadherin peptidic chain, the sixteenth, which is proline in humans and glutamic acid in mice. This result led to the conclusion that *L. monocytogenes* exhibits a species-specificity towards its host, and that the mouse model is inappropriate to study internalin function (Lecuit et al., 1999). Similarly, InlB was shown to be species-specific being able to promote entry and ruffling in human and mice cells but not in guinea pig and rabbit cells. As a consequence, no role could be demonstrated for

InlB in guinea pigs and rabbit infections although it played a role in liver and spleen colonization in mice (Khelef et al., in press).

A more appropriate animal species for studying the putative role of internalin interaction with E-cadherin in vivo turned out to be the guinea pig. Indeed, internalin-dependent entry of *L. monocytogenes* was demonstrated in cultured guinea pig cells expressing E-cadherin, harbouring a proline at position 16 as human E-cadherin (Lecuit et al., 1999). Moreover, induction of a gastroenteritis by *L. monocytogenes* resembling that observed in humans was observed in the guinea pig (Racz et al., 1972; Dalton et al., 1997; Pron et al., 1998; Aureli et al., 2000; Lecuit et al., 2001b). In addition, it is able to cross the intestinal barrier and induce a dose- and internalin-dependent lethality following dissemination into the systemic circulation (Lecuit et al., 2001b). Yet, this model remains inappropriate to study concomitantly the role of both InlA and InlB. To investigate the role of the internalin-E-cadherin interaction in the ability of *L. monocytogenes* to cross the intestinal barrier, a transgenic mouse model was generated. The human E-cadherin cDNA was placed under the control of the promoter of the intestinal fatty acid binding protein (iFABP) gene, which is exclusively active in post-mitotic nonproliferative small intestinal enterocytes. In this transgenic model, *L. monocytogenes* directly targets enterocytes by interacting with enterocytic E-cadherin. This interaction leads to *L. monocytogenes* internalization into these cells and allows the subsequent crossing of the intestinal barrier, followed by bacterial multiplication in the small intestine lamina propria, and dissemination to mesenteric lymph nodes, liver and spleen (Fig. 48). This is the first transgenic model to reveal the role of a bacterial virulence factor and to demonstrate its critical implication in a key step of an infection process. The enterocyte-restricted human E-cadherin expression has been critical to demonstrating at a molecular level the direct in vivo targeting of enterocytes by *L. monocytogenes* and the genuine enteropathogenicity of *L. monocytogenes*. Furthermore, this transgenic model not only provides the molecular explanation for the innocuity of *L. monocytogenes* in mice following oral infection but also explains the enteropathogenicity of *L. monocytogenes* in guinea pigs, and most probably in humans (Lecuit et al., 2001b). A mouse model for orally acquired listeriosis is now available. The host response to listeriosis can now be studied in depth from its starting point (the intestinal lumen) using the combined approaches of microbial genetics, transgenesis, gnotobiology, and functional genomics, coupled to laser capture microdissection, as described in the study of host
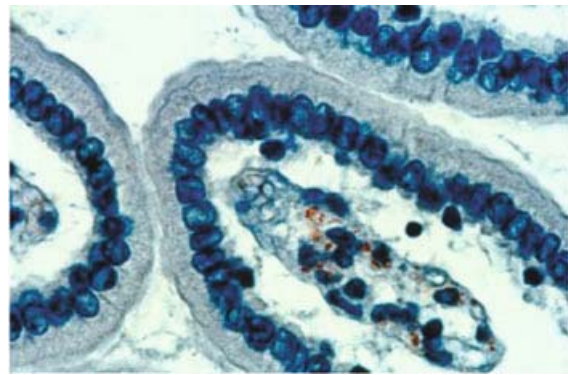
Fig. 48. *Listeria monocytogenes* multiplication within the lamina propria of a small intestinal villus of an *iFABP-hEcad* (intestinal fatty acid binding protein-human E-cadherin) transgenic mouse 48 h after intragastric inoculation. A section through the small intestine of an *iFABP-hEcad* transgenic mouse is immunolabeled with rabbit anti-*L. monocytogenes* R11 polyclonal antibody and the *Listeria* are revealed with anti-peroxidase antibodies (red). *Listeria monocytogenes* have crossed the intestinal barrier and replicate in the lamina propria, a phenomenon that is never observed in nontransgenic mice. Scale bar 10 μm. From Lecuit et al. (2001b), with permission.

responses to commensal bacteria by Hooper and Gordon (2001). The availability of the *L. monocytogenes* genome, together with that of its nonpathogenic counterpart *L. innocua*, will probably be very helpful in identifying additional virulence factors in this new model. This system, however, as the models for human viral infections presented above, has its limits. These were anticipated when the transgenesis strategy was designed. Indeed, since human E-cadherin expression is restricted to enterocytes in the *iFABP*-human E-cadherin transgenic mice, the role of other cell types expressing E-cadherin (such as dendritic cells, hepatocytes, microvascular endothelial cells, epithelial cells of the choroid plexus, and cytotrophoblastic cells, all of which are putative *L. monocytogenes* targets during human listeriosis) cannot be addressed. Mice overcoming this limitation are now being generated by changing the codon for glutamic acid 16 of mouse E-cadherin at the endogenous mouse E-cadherin locus *Cdh1* on chromosome 8 into a codon for proline. This unique change in murine E-cadherin has indeed been shown to be sufficient to convert mouse E-cadherin into an internalin receptor in transfected cultured cells expressing this modified Glu16Pro mouse E-cadherin (Lecuit et al., 1999). The new transgenic mouse line should permit the study of the tropism of *L. monocytogenes* for the CNS and the feto-placental unit (which is responsible for the lethality of human listeriosis) and to further investigate the role of internalin E-cadherin interaction in extraintestinal tissues. This model

should also allow addressing the role of InlB in mice after oral infections. The importance of these future studies is emphasized by the results of our recent epidemiological investigations, which showed that 100% of *L. monocytogenes* isolates obtained from pregnancy-associated listeriosis and collected in a one-year period express a functional internalin, whereas only 65% of food isolates collected during the same period express functional internalin. These results strongly argue in favor of a role for internalin in crossing the maternofetal barrier (Jacquet et al., 2004). This hypothesis has now been confirmed experimentally (Lecuit et al., 2004). Recent studies using different *L. monocytogenes* strains reveal that, in contrast to strains from other serovars, serotype 4b epidemic strains appear to be able to cause systemic infection in mice infected orally. This suggests that serovar-specific virulence factors might be playing a role in mouse susceptibility to orally acquired listeriosis. These observations highlight that the properties of an animal model may vary from strain to strain of a single bacterial species (Czuprynski et al., 2003).

Comparative Genomics

Comparative genomics and related technologies are helping to unravel the molecular basis of pathogenesis, host range, evolution and phenotypic differences of Listeriae. The first application of genomics to *Listeria* research was the determination by a consortium of 10 European laboratories of the complete genome sequences of the pathogen *L. monocytogenes* (strain EGDe) and the closely related nonpathogenic species *L. innocua* (strain CLIP11626) (Glaser et al., 2001). The availability of these two sequences (see ListiList Worldwide Web Server (http://genolist.pasteur.fr/ListiList/)) opens the way to comparative and functional genomics and the use of new approaches like bioinformatics, microarrays and proteomics to gain functional information (Fig. 49). Additional sequence information is available as a second *L. monocytogenes* isolate, strain F2365 (Scott A), is being sequenced by the TIGR {Technical Institute of Genomic Research} in collaboration with the USDA. Furthermore, the determination of the genome sequences of *L. ivanovii*, *L. seeligeri*, *L. welshimeri* and *L. grayi* strains by the German PathoGenomik network in collaboration with the Institut Pasteur are nearing completion. The availability of these different genome sequences of Listeriae will pave the way for in-depth comparative genomics and the identification of unknown virulence determinants.

THE GENOME SEQUENCE OF *L. MONOCYTOGENES* EGDe AND *L. INNOCUA* CLIP11262 The *L.*
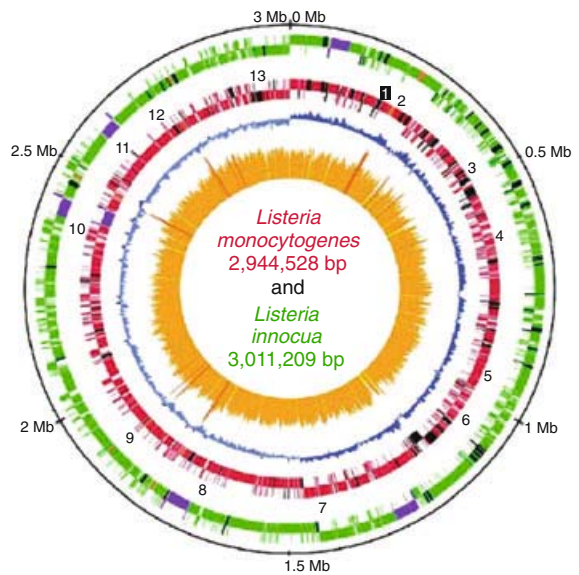
Fig. 49. Circular genome maps of *L. monocytogenes* EGD-e and *L. innocua* CLIP 11262 showing the position and orientation of genes. Circle 1 and 2, *L. innocua* and *L. monocytogenes* genes on the + and – strands, respectively. *Listeria innocua* genes, green; *L. monocytogenes* genes, red; genes specific for *L. monocytogenes* or *L. innocua*, black; rRNA operons, orange; and prophages, purple. Numbers on the second circle indicate the position of known virulence genes: 1, virulence locus (*prfA-plcA-hly-mpl-actA-plcB*); 2, *clpC*; 3, *inlAB*; 4, *iap*; 5, *dal*; 6, *clpE*; 7, *lisRK*; 8, *dat*; 9, *inlC*; 10, *arpJ*; 11, *clpP*; 12, *ami*; and 13, *bvrABC*. Circle 3, G/C bias (G1C/G–C) of *L. monocytogenes*. Circle 4, G1C content of *L. monocytogenes* (32.5% G1C in light yellow, 32.5–43.5% in yellow, and >43.5% G1C in dark yellow). The scale in megabases is indicated on the outside of the genome circles, with the origin of replication at position 0. From Glaser et al. (2001), with permission.

monocytogenes EGDe genome comprises 2,944,528 bp with an average G+C content of 39% and 2853 predicted protein-coding genes. The *L. innocua* CLIP11626 chromosome is 3,011,209 bp long with an average G+C content of 37% and 2981 predicted protein-coding genes (Glaser et al., 2001). Analysis of the two *Listeria* genomes allowed common and particular features of Listeriae to be determined, as well as differences between a pathogenic and a nonpathogenic *Listeria* strain. One interesting common feature is the finding that 2587 of the 2853 *L. monocytogenes* genes have an orthologue in the *L. innocua* genome (Glaser et al., 2001). Furthermore, a perfect conservation of the order as well as the relative orientation of these orthologous genes was identified, indicating a high stability in the genome organization of Listeriae and a close phylogenetic relationship of the two *Listeria* genomes (Buchrieser et al., 2003). However, despite this high number of common genes, considerable differences in gene content exist

between the two *Listeria* genomes, some of which are undoubtedly related to the ability of *L. monocytogenes* to cause disease in humans and animals.

A particular feature of the *Listeria* genomes is the presence of an exceptionally large number of surface proteins, as 4.8% of all predicted genes of *L. monocytogenes* EGDe and 4.3% of the *L. innocua* genome code for surface proteins. Interestingly, the differences between the two *Listeria* genomes are also the most pronounced within genes encoding surface proteins, in particular among those that encode proteins possessing a peptidoglycan anchoring domain (LPXTG motif) (Navarre and Schneewind, 1999). The *L. monocytogenes* EGDe genome encodes 41 such proteins, 19 of which are absent from the *L. innocua* CLIP11626 genome. In contrast *L. innocua* contains 34 LPXTG proteins, 14 of which are *L. innocua* CLIP11626 specific (Glaser et al., 2001; Cabanes et al., 2002). The difference in surface proteins may be related to the additional capacity of *L. monocytogenes* to interact with various eukaryotic cell types.

Besides surface proteins, an abundance of transport proteins (331 genes in *L. monocytogenes* EGDe and 313 in *L. innocua* CLIP11626) and an extensive regulatory repertoire (209 genes in the *L. monocytogenes* EGDe and 203 in *L. innocua*) are characteristic for the *Listeria* genomes. These data correlate with the capacity of *Listeria* to adapt and respond to a wide variety of different environments and to its property to colonize a broad range of ecosystems. A high percentage of the encoding capacity of the two *Listeria* genomes is dedicated to transport proteins (11.6% and 11.4%, respectively) and to regulatory proteins (7.3% and 7.1%, respectively). However, the differences between the two *Listeria* genomes are not as pronounced as the differences identified in the surface protein repertoire, suggesting their implication primarily in specific features common to the lifestyle of Listeriae outside a mammalian host.

SPECIES-SPECIFIC GENES AND STRAIN-SPECIFIC GENES: A GLOBAL VIEW The genome comparison of *L. monocytogenes* EGDe and *L. innocua* CLIP11626 identified a conserved genome organization and a high number of orthologous genes, but it also revealed the presence of 270 *L. monocytogenes* EGDe-specific genes (9.5%) and 149 *L. innocua* CLIP11626-specific genes (5%), when prophages were not taken into account. The *L. monocytogenes* EGDe-specific genes are present in 100 DNA fragments scattered throughout the entire chromosome, and the *L. innocua* CLIP11626 specific genes are clustered in 63 regions containing 1–7 genes (Glaser et al., 2001; Buchrieser et al., 2003). This particular

organization of a number of small regions within the *Listeria* genomes suggests that multiple acquisition, but also deletion events, have led to the present genome content. The question arises now whether these genes (e.g., the 41 genes of *L. monocytogenes* EGDe encoding for surface proteins with a LPXTG motif) are consistently present in all *L. monocytogenes* isolates or whether further differences exist among the different *L. monocytogenes* strains. To this end additional sequence information and comparative genomic approaches using the DNA-array technique were employed. *Listeria monocytogenes* strains belonging to different sub-groups other than the EGDe strain (serovar 1/2a), e.g., serovar 4b strains, exhibit considerable differences in their gene content. Indeed, about 8% of the genes are specific to this group of strains but absent from *L. monocytogenes* serovar 1/2a (Doumith et al., 2004a). These results are in line with a previous report (Herd and Kocks, 2001) that identified 39 specific gene fragments for the epidemic *L. monocytogenes* strain F4565 as compared to *L. monocytogenes* EGDe using a subtractive hybridization method. When comparing genes encoding for different protein families among the three *Listeria* strains sequenced (*L. monocytogenes* EGDe, *L. monocytogenes* serovar 4b, and *L. innocua* CLIP11262), the importance of the surface protein encoding genes, in particular those with a LPXTG anchor motif, was clearly underlined. Genes encoding proteins belonging to the LPXTG family showed the most pronounced differences among the strains. We identified a core gene pool of 20 genes encoding for LPXTG proteins and specific ones in each strain. This indicates that this protein family might be strongly implicated in strain-specific and species-specific features of Listeriae. These findings were further substantiated by the results obtained from DNA-DNA hybridization of 93 *L. monocytogenes* strains and 20 strains belonging to the remaining five species of the genus *Listeria* using DNA-arrays (Doumith et al., 2004a). Again, the distribution of surface-protein encoding genes was very heterogeneous. However, each sub-group within the species *L. monocytogenes* was characterized by the presence of specific surface protein-coding genes and only a small group is consistently present in all the *L. monocytogenes* isolates tested. For instance, differences among genes encoding phosphotransferase system (PTS) components or regulatory proteins were much less pronounced, suggesting that they account less for intra- or inter-species differences (Doumith et al., 2004a). These results further provide an explanation why previous studies have found an association between various characteristics of *L. monocytogenes* and serovars, leading to the def-

inition of three lineages within the species *L. monocytogenes*, which are correlated with serovars, suggesting that this association is due to an evolutionary differentiation.

Analysis of the hybridization profiles of different strains revealed distinct patterns of gene presence and absence in different subgroups of *L. monocytogenes* and also allowed identification of 30 *L. monocytogenes*-specific and several serovar-specific marker genes (Doumith et al., 2004a). Based on these results, four serovar-specific marker genes were selected and exploited to design a multiplex PCR, allowing the differentiation of the major *Listeria monocytogenes* serovars (Doumith et al., 2004b). These patterns and markers should further prove to be powerful tools for the rapid tracing of listeriosis outbreaks by PCR for instance, but they also provide a basis for the functional study of virulence differences between *L. monocytogenes* strains. These marker genes and the specific primers are available at Website of Genomic of Microbial Pathogens Unit at Institut Pasteur (http://www.pasteur.fr/recherche/unites/gmp/sitegmp/biodiversitylist. html).

A similar approach using a shotgun microarray and DNA-DNA hybridization corroborated these results by identifying genes specific for *L. monocytogenes* serovar 1/2a as compared to *L. monocytogenes* serovar 4b and 1/2b strains (Zhang et al., 2003). Based on *prf*A virulence gene cluster sequences from 113 *L. monocytogenes* isolates, the presence of three evolutionary lineages among the species *L. monocytogenes* was confirmed and it was shown that the human epidemic associated serotype 4b is prevalent among strains from lineage 1 and lineage 3 (Ward et al., 2004). These results also allowed to develop a PCR-based test for lineage identification.

These different genomic approaches applied to the genus *Listeria* provide for the first time a global view and a more complete knowledge of the gene distribution and the genetic content present in the gene-pool of the genus *Listeria*. This information represents a fundamental basis for functional studies to better understand phenotypic and virulence differences between *L. monocytogenes* strains.

Unsolved Questions and Concluding Remarks

In nearly two decades, *Listeria* has become one of the best-documented intracellular bacteria. Our knowledge of the bacterial factors contributing to infection is increasing daily. Yet understanding the function of the many factors encoded by the genome, involved or not in virulence, will require years of work. When are they expressed, to which regulators are they subjected, when are they active? What is the function of the intergenic regions? Are there some small RNAs involved in virulence? All these questions will require the help of system biology and large-scale genomic analysis.

Several groups are also now addressing biodiversity issues. It is clear that we do not have yet an answer to the question of why some strains are more epidemic than others. However, this information should be available soon, and genes and factors absolutely required for human infection, in addition to the well-established virulence markers LLO and ActA, should be identified soon and fully characterized. Information will also come from large epidemiological studies such as those showing that a complete and functional internalin is as critical as LLO for pathogenesis in humans (Jacquet et al., 2004; Rousseaux, 2004). More interestingly, all serovar 4b strains have a functional internalin, which is not the case for strains of other serovars (Jacquet et al., 2004).

A point that remains unclear is what the infective dose for humans? This is unknown and obviously depends on many individual factors and in particular on the immune status of the host. While ongoing research on adaptive immunity to *Listeria* infections is highly documented (Lara-Tejero and Pamer, 2004), efforts to understand the innate response to *Listeria* are now a priority.

Progress was accomplished to understand how *Listeria* cross barriers. We now know that internalin is used to cross both the intestinal and placental barriers. How does *Listeria* cross the blood brain barrier? There are arguments to predict that internalin may also be used, but the ad hoc animal model is not yet available.

Listeria is one of the "stars" in cellular microbiology (Cossart and Sansonetti, 2004). Its behavior, when in contact with the cell or when spreading intra- and intercellularly, is one of the most sophisticated bacterial behaviors. Many cellular factors contributing to infection have been identified. It is clear that what is lacking now is the spatiotemporal description of all these events at the cellular level, as well as in vivo. All the new in vivo imaging techniques are there. We just need to use them!

In conclusion, it is good that this chapter can be electronically corrected. We are at a period where information is constantly changing, and we still have a lot to learn by studying this amazing organism.

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CHAPTER 1.2.12

The Genus *Brochothrix*

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The genus *Brochothrix* contains Gram-positive, nonsporeforming, nonmotile, catalase-positive, facultatively anaerobic, regular, rod-shaped bacteria that show characteristic changes in cell morphology during growth. The genus was proposed by Sneath and Jones (1976) for some meat spoilage organisms, previously designated "*Microbacterium thermosphactum*" (McLean and Sulzbacher, 1953). The genus *Brochothrix* contains the type species *B. thermosphacta* (see Sneath and Jones, 1986) and *B. campestris* Talon et al. (1988), described for isolates from soil and grass. As most of the scientific interests have focused on *B. thermosphacta* because of its association with off-odor development in meats, especially in prepacked products held at refrigeration temperatures, most of the information on the genus is consequently derived from studies on this species. There is no evidence that any *Brochothrix* strain is pathogenic to humans or animals. Neither species has been exploited in industrial processes.

The type strain of *B. thermosphacta* is ATCC [{www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=11509}{11509}]^T (DSM 20171^T) and that of *B. campestris* is CIP [{www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=10290}{10290}]^T = S$_3$^{T} (DSM 4712^T).

Phylogeny and Classification

The bacteria were first isolated and described by Sulzbacher and McLean (1951) during studies on pork sausage meat and were later allocated to the genus *Microbacterium* as a new species, *M. thermosphactum* (McLean and Sulzbacher, 1953). Their classification in the genus *Microbacterium* was due largely to the then poor circumscription of that genus (see Keddie and Jones, 1981). McLean and Sulzbacher (1953) noted the marked difference in cell morphology between *M. thermosphactum* and *M. lacticum*, the type species of the genus, and also commented on the close physiological resemblance between *M.*

thermosphactum and the lactobacilli. However, at that time, both *Microbacterium* and *Lactobacillus* were classified in the family Lactobacteriaceae, and the main distinction between the two genera was catalase production (Breed et al., 1948), therefore, McLean and Sulzbacher (1953) assigned their catalase-positive isolates to the genus *Microbacterium*.

Later workers confirmed and augmented the differences between *M. thermosphactum* and *M. lacticum*, not only in cell morphology (Davidson et al., 1968b; Jones, 1975) but also in enzymology and protein profiles (Robinson, 1966; Collins-Thompson et al., 1972), in peptidoglycan structure (Schleifer, 1970; Schleifer and Kandler, 1972), and in DNA base composition (Collins-Thompson et al., 1972). In addition, numerical taxonomic studies showed that *M. thermosphactum* strains formed a relatively homogeneous taxon (intra-group similarity greater than 85%) quite distinct from *M. lacticum* (Davis and Newton, 1969a; Davis et al., 1969b; Jones, 1975; Wilkinson and Jones, 1977). The same studies indicated that the closest associates of *M. thermosphactum* were the genera *Listeria* and *Lactobacillus*, and in one study, *Kurthia* (Davis and Newton, 1969a). In none of the studies, however, were the similarities close enough to justify the inclusion of *M. thermosphactum* as a new species in any of these genera. Consequently, Sneath and Jones (1976) concluded that *M. thermosphactum* strains were sufficiently distinct from other Gram-positive bacteria to merit a separate genus and proposed that they be reclassified in a new genus *Brochothrix* as *B. thermosphacta*. These authors were aware that the taxonomic relatedness of *Brochothrix* to other Gram-positive bacteria at the suprageneric level was problematic. After evaluating all the data then available, including the presence of catalase and cytochromes in *B. thermosphacta* (Davidson and Hartree, 1968a; Davidson et al., 1968b) and the reported difference in fatty acid composition between *Brochothrix* and *Lactobacillus* (Shaw and Stead, 1970), they tentatively placed *Brochothrix* in the family Lactobacillaceae (Bucha-
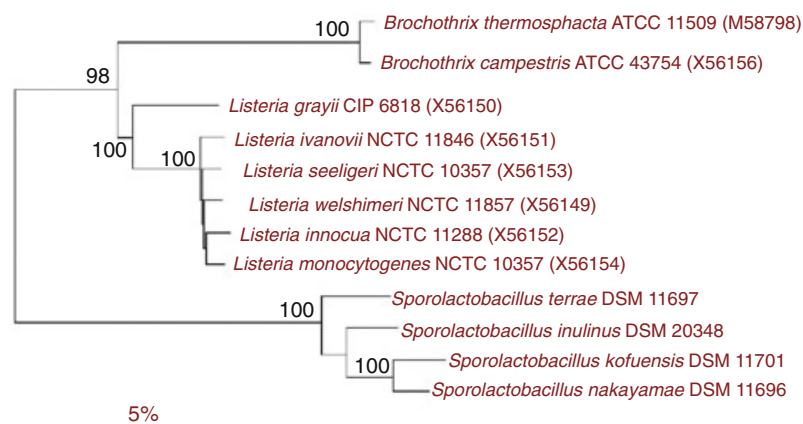
Fig. 1. Dendrogram of 16S rRNA gene similarities (De Soete, 1983) between type strains of species of the genera *Brochothrix*, *Listeria* and *Sporolactobacillus*. Numbers indicate the percentage of bootstrap samplings, derived from 1000 resamplings (Felsenstein, 1993). Bar indicates 5% sequence divergence. Numbers in parentheses are accession numbers of 16S rRNA gene sequences. The sequences of *S. terrae*, *S. kofuensis* and *S. nakayamae* were determined for this revision.

nan and Gibbons, 1974); this family included the genus *Lactobacillus* and three other genera designated as having uncertain affiliation, i.e., *Listeria*, *Erysipelothrix* and *Caryophanon*.

Subsequently, it became apparent that in phenotype, the members of the genus *Brochothrix* more closely resembled those of the genus *Listeria* than those of the genus *Lactobacillus* (see Sneath and Jones, 1986). Both *B. thermosphacta* and *Listeria* spp. possess catalase and cytochromes (Davidson and Hartree, 1968a; Feresu and Jones, 1988). They also contain *meso*-diaminopimelic acid in the cell wall peptidoglycan (Schleifer and Kandler, 1972), possess menaquinones with seven isoprene units (MK-7) as the predominant isoprenoid quinone (Collins et al., 1979; Collins and Jones, 1981; Feresu and Jones, 1988), and contain predominantly methylbranched chain fatty acids (Shaw, 1974; Feresu and Jones, 1988). This close phenotypic similarity between *B. thermosphacta* and *Listeria* spp. was confirmed by the results of the 16S rRNA oligonucleotide sequencing studies of Ludwig et al. (1984). These studies showed that *B. thermosphacta* and *L. monocytogenes* are phylogenetically very closely related and form one of the several sublines within the *Bacillus-Lactobacillus-Streptococcus* cluster of the clostridial subbranch of the Gram-positive eubacteria (Stackebrandt and Woese, 1981; Collins et al., 1991).

The species *B. campestris* was named by Talon et al. (1988) on the basis of numerical taxonomic and DNA hybridization studies of a number of strains of *Brochothrix* spp. isolated from a variety of sources, including grass and soil.

On the basis of analyses of 16 rRNA gene sequences, *Brochothrix thermosphacta* and *B. campestris* are highly related, sharing 99.3% sequence similarity. Their phylogenetic neighbors are members of the genus *Listeria* (92.8–96.6%) and less closely related members of the genus *Sporolactobacillus* (<92% on the basis of
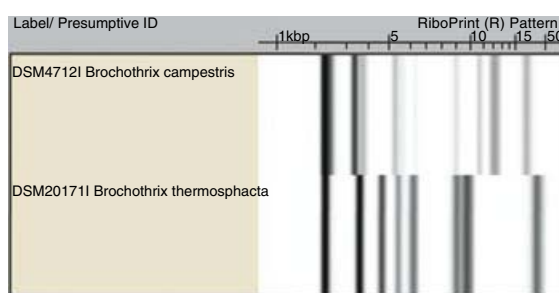
Fig. 2. Diversity of normalized *Eco*RI ribotype patterns found within the two type strains of *Brochothrix* species.

ARB [from Latin arbor, tree] analysis; Ludwig et al., 2004). As depicted in the phylogenetic tree (De Soete, 1983; Fig. 1), the three genera are phylogenetically well separated. The small phylogenetic distance found between the two *Brochothrix* type strains are also seen among some of the *Listeria* type strains, i.e., *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, *L. innocua* and *L. monocytogenes* (>99.1%). The majority of the branching points are supported by high bootstrap values.

As determined by the S1 nuclease method at 60°C, *B. campestris* strains exhibit about 15% DNA similarities to *B. thermosphacta* (Talon et al., 1988), which identifies both strains as sound genomospecies. This is also reflected by the significant difference in the RiboPrint patterns (Fig. 2).

Chemotaxonomy

Other than the presence of *meso*-diaminopimelic acid in the cell wall of *Brochothrix campestris* (Talon et al., 1988) and *B. thermosphacta* (Schleifer, 1970; Schleifer and Kandler, 1972), all the information on chemical composition is based on studies with *B. thermosphacta*. Arabinose and galactose are not present in the cell wall (see

Schleifer and Kandler, 1972), nor are mycolic acids (Minnikin et al., 1978; Feresu and Jones, 1988). The major fatty acid is 12-methyltetradecanoic (*anteiso*-C$_{15:0}$ with substantial amounts of 14-methylhexadecanoic (*anteiso*-C$_{17:0}$) and 13-methyltetradecanoic (*iso*-C$_{15:0}$) acids (Shaw and Stead, 1970; Feresu and Jones, 1988). The major phospholipids are phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine. The glycolipid fraction contains an acylated glucose and small amounts of a glycosyl diglyceride tentatively identified as dimannosyl diglyceride (Shaw and Stead, 1970; Shaw, 1974). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) whole-cell protein profiling has been used to characterize *B. thermosphacta* during monitoring changes in populations in chilled vacuum-packed beef (Sakala et al., 2002)

Brochothrix thermosphacta and *B. campestris* contain several membrane-bound respiratory cytochromes, such as *aa*$_3$, *a*$_3$, *b* and *d* (Gil et al., 1992), but only strains of the former species were subjected to analyses at different growth temperatures. Under moderate aeration, cytochromes *b* and *d* dominate at 30°C, while *aa*$_3$ and *a*$_3$ are the dominating components when grown at 10°C and 15°C. Reduction of the oxygen content in batch cultures lead to an enhancement of cytochrome *d* and a reduction in *a*-type cytochromes. Cytochrome *c* was missing in both species, which otherwise show similar cytochrome compositions when grown at 10°C.

Menaquinones are the sole respiratory quinones; MK-7 is the major component, and MK-6 and MK-5 are minor components (Collins et al., 1979; Collins and Jones, 1981; Feresu and Jones, 1988).

The G+C content of the DNA of *B. thermosphacta* is 36–37 mol% (Collins-Thompson et al., 1972; Collins et al., 1987; Feresu and Jones, 1988) and of *B. campestris*, 38 mol% (Talon et al., 1988).

Characterization

The colonial morphology of *Brochothrix* spp. is not particularly diagnostic. After 24–48 h, the colonies are circular, 0.75–1 mm in diameter, convex with entire margins, and not pigmented. In young cultures of *B. thermosphacta*, two types of colony varying in size and density may be present. These can be so distinct that the culture may appear to be contaminated (see Barlow and Kitchell, 1966). In older cultures of the same species, the edge of the colony breaks up and the center becomes raised to give a "fried egg" appearance. Neither of these two phenomena has been reported for *B. campestris* (Talon et al., 1988). *Brochothrix* spp. are nonhemolytic, but sometimes an area of weak greening is apparent around colonies on blood agar.

Gram-stains on exponential-phase cultures of *B. thermosphacta*, performed on 18–24 h and 2-day cultures grown on nutrient agar such as blood agar base (Columbia or BAB no. 2, Oxoid) or APT medium incubated at 20–25°C, show regular, unbranched rods that occur singly in pairs and short chains and in long, kinked, filamentous-like chains (Fig. 3a) that bend and loop to give characteristic knotted masses. This phenomenon has not yet been reported for *B. campestris*; in this species 24-h cultures are reported to consist of a mixture of long and short rods that usually occur singly or in pairs (Talon et al., 1988). In older cultures of *B. thermosphacta*, the rods give rise to coccoid forms (Fig. 3b) that when subcultured onto a suitable medium develop into rod forms. Both rod and coccal forms are Gram-positive, but a proportion may appear Gram-negative. They are nonmotile and do not form endospores or capsules. Talon et al. (1988) reported that about one-half of the *B. thermosphacta* strains they examined produced slime from sucrose in broth culture.
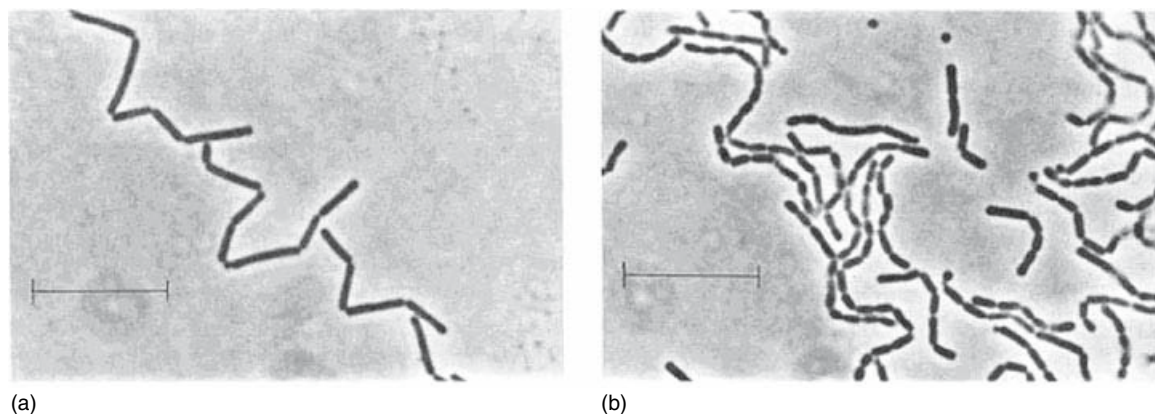
Fig. 3. *Brochothrix thermosphacta* isolate, grown on blood agar base no. 2 (Oxoid) at 25°C. (a) After 24 h, showing regular rods in chains. (b) After 48 h, showing development of coccoid forms. Bar = 10 μm.

In MRS medium the type strains of *B. thermosphacta* and *B. campestris* produce acid fermentatively from glucose, ribose, fructose, mannose, salicin, maltose, D-cellobiose, mannitol, trehalose and, weakly, from gluconate (R. Pukall, unpublished observation); acid production from *N*-acetylglucosamine has also been reported; they are methyl-red and Voges-Proskauer positive. Esculin is hydrolyzed. Exogenous citrate and urea are not utilized. Indole and H$_2$S are not produced, nitrate is not reduced, gelatin is not liquefied, and arginine is not hydrolyzed (McLean and Sulzbacher, 1953; Sneath and Jones, 1976; Wilkinson and Jones, 1977; Talon et al., 1988).

With use of the API ZYM test system (API System, La Balme des Grottes, France), both species are reported to produce the following arylamidases: phenylalanine, histidine, glycyl-phenylalanine, seryl-tyrosine, glutamate, tryptophan, and histidyl-L-phenylalanine (Talon et al., 1988).

In the API esterase tests, Talon et al. (1988) noted that all five strains of *B. campestris*, but only 12 of 165 strains of *B. thermosphacta* tested, hydrolyzed naphthylbutyrate, naphthylvalerate, naphthylcaproate, naphthylcaprylate, naphthylnonanoate and naphthylcaprate.

According to the BIOLOG GP2 substrate panel (R. Pukall, unpublished observation), *B. thermosphacta* DSM [{www.ncbi.nlm.nih.gov/entrez /query.fcgi?db=nucleotide=search=20171} {20171}]^T and *B. campestris* DSM [{www.ncbi.nlm.nih.gov/entrez/query.fcgi? db=nucleotide=search=4712}{4712}]^T utilize dextrin, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, amygdalin, arbutin, D-cellobiose, D-fructose, gentiobiose, D-gluconic acid, μ-D-glucose, *m*-inositol, maltose, maltotriose, D-mannitol, D-mannose, pyruvic acid, palatinose, D-trehalose, thymidine, β-methyl-D-glucoside, D-ribose, salicin, glycerol, inosine and uridine. Neither type strain utilizes α-cyclodextrin, β-cyclodextrin, glycogen, inulin, mannan, Tween 40, Tween 80, L-arabinose, D-arabitol, L-fucose, D-galactose, D-galacturonic acid, α-D-lactose, lactulose, D-melizitose, D-melibiose, D-tagatose, xylitol, D-xylose, acetic acid, α- and β-hydroxy-butyric acid, γ-hydroxy-butyric acid, p-hydroxy-phenyl acetic acid, α-keto-glutaric acid, lactamide, D-lactic acid methyl ester, D- and L-malic acid, mono-methyl succinate, propionic acid, succinamic acid, succinic acid, N-acetyl-L-glutamic acid, L-alaninamide, D- and L- alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, glycyl-L-glutamic acid, L-pyroglutamic acid, α- and β-methyl-D-galactoside, 3-methyl-glucose, α-methyl-D-glucoside, α-methyl-D-mannoside, D-raffinose, sedoheptulosan, D-sorbitol, stachyose, L-serine, putrescine, 2,3-butandiol, 2'deoxy adenosine, adenosine-5'monophosphate, thymidine-5'monophosphate, uridine-5'monophosphate, fructose-6-phosphate, glucose-1-phosphate, and glucose 6-phosphate.

Both species are susceptible to a wide range of antibiotics. Using antibiotic disks on Mueller-Hinton agar, Talon et al. (1988) reported all strains of *B. thermosphacta* (104 tested) and *B. campestris* (5 tested) were sensitive to novobiocin, tetracycline (except for three strains of *B. thermosphacta*), amikacin, tobramycin, gentamicin, and ampicillin and were resistant to oxacillin, nalidixic acid, and colistin (except the type strain of both species and one other strain of *B. campestris*). A wider range of antibiotics was tested with *B. thermosphacta* (15 strains) on nutrient agar medium by Feresu and Jones (1988).

Further diagnostic information on both species is given in Table 1. Both type strains are suitable reference strains, but it should be noted that *B. campestris* grows with 8% NaCl if incubated for 6 days (Talon et al., 1988).

Sneath and Jones (1976) reported that *B. thermosphacta* does not produce deoxyribonuclease, but later studies have detected both deoxyribonuclease and ribonuclease activity in strains of this species (Wilkinson and Jones, 1977; Feresu and Jones, 1988).

The results of many of the conventional tests can be influenced markedly by the media and methods used. Differences in methodology probably account for some of the discrepancies noted in the literature. Media and methods such as those used by Talon et al. (1988) and Wilkinson and Jones (1977) are suitable for most tests, and incubation should be at 20–25°C.

Differentiation of Brochothrix species

The most useful phenotypic characters for differentiating the two species are growth in the presence of 8 and 10% NaCl, growth on BM medium (Wilkinson and Jones, 1977) containing 0.05% potassium tellurite, hippurate hydrolysis, and production of acid from rhamnose (Table 1).

Molecular Identification

Affiliation of a yet unassigned isolate to the genus is unambiguously done by sequence analysis of the 16S rRNA gene when sequence differences between the type strains of the two species are significant (Stackebrandt and Goebel, 1994). The difference between the type strains of *Brochothrix* is 0.7% only, making it difficult to taxonomically place a new strain. A

Table 1. Additional and differential features of *Brochothrix* spp.

Features	B. thermosphacta	B. campestris
Growth with		
NaCl, 8% (2 days)	+	−
NaCl, 10% (2–7 days)	d	−
Growth with and reduction of potassium tellurite, 0.05%	+	−
Reduction of tetrazolium, 0.01%	d	+
Slime produced from sucrose	d	−
Resistance to furadoine	d	−
Hydrolysis of:		
Hippurate	−	+
Cellulose	+	ND
Tyrosine	−	ND
Xanthine	−	ND
Production of		
Phosphatase	+	ND
Sulfatase	−	ND
Utilization according to BIOLOG GP2		
Turanose	+	−
α-Keto valeric acid	+	−
L-Lactic acid	+	−
Rhamnose	−	+
Saccharose	+	−
Adenosine	+	−
D,L-α-glycerol-phosphate	+	−
Acid from		
Amygdalin	+	+
Arbutin	d	+
Dulcitol	+	ND
Gentobiose	+	d
Glycerol	−	+
Inositol	+	d
Inulin	d	ND
Lactose	+[a]	ND
Mannitol	+	d
Melezitose	d[a]	−[a]
Melibiose	d[a]	−[a]
Rhamnose	−	+
Sorbitol	d[a]	−[a]
Sorbose	−	ND
Starch	−	d
Sucrose	+	d[a]
Tagatose	+	d
Xylose	d	ND

Symbols and abbreviations: +, 90–100% of strains positive; −, 0–10% of strains negative; d, 11–89% of strains positive; ND, no data.

[a]Type strain negative in MRS. (de Man-Rogosa-Sharpe).

strain branching deeper than the bifurcation of the two type strains but closer to these than to members of the nearest neighboring genus must be taxonomically evaluated by means of genus-specific properties to decide whether it belongs to *Brochothrix* or whether it forms the nucleus of a novel genus.

Riboprints are available for the two type strains (Fig. 2), but patterns from more strains of both species are needed to evaluate the homogeneity of rrn cistron cleaving patterns of these taxa. Oligonucleotide primers targeting variable regions within the 16rRNA gene have been designed for the rapid detection of *Brochothrix*

spp. (Grant et al., 1993). When combined with the capture of *Brochothrix* cells using a lectin immobilized on magnetic beads the polymerase chain reaction (PCR) assay targeting DNA from released cells was sufficiently sensitive to detect as low as about 100 colony forming units (CFU) per gram of chopped fresh chicken meat.

A rabbit polyclonal antibody-linked probe allowed detection of 76% of 800 presumptive *B. thermosphacta* isolates from British fresh sausages. Of collection strains investigated, only the type strain NCTC [{www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=10822}{10822}][T] did not react, while one strain each of

Kurthia zopfii and *Staphylococcus aureus* reacted positively, demonstrating the lack of specificity (Stringer et al., 1995). Other detection methods (such as denaturing gradient gel electrophoresis [DGGE; Cocolin et al., 2001], oligonucleotide probes [Kim et al., 1991], and low molecular weight RNA profiles) were able to differentiate lactobacilli and *Listeria* spp. from *Brochothrix* spp. and other meat spoilage organisms (Collins-Thompson et al., 1991).

Differentiation of Brochothrix from Phylogenetically Neighboring Genera

Phenotypic identification of new isolates as *Brochothrix* spp. requires examination of cellular morphology and staining reactions, the maximum growth temperature, and relationships to oxygen and catalase production together with a number of conventional taxonomic tests, such as the ability to produce acid from various sugars. The chemical composition of the organisms also aids the identification of *Brochothrix* spp., but performing the relevant analyses for routine identification is not usually necessary. Features that differentiate the genus from other Grampositive, nonsporeforming, rod-shaped bacteria are listed in Table 2.

Brochothrix spp. are readily distinguishable from the morphologically similar members of the genus *Kurthia* by examining the oxygen requirements. *Brochothrix* is facultatively anaerobic whereas *Kurthia* is strictly aerobic. In addition, *Brochothrix* spp. produce acid from a wide variety of sugars. Most strains of *Kurthia* are highly motile.

The inability of *Brochothrix* spp. to grow at 35°C distinguishes them from members of the genera *Carnobacterium*, *Erysipelothrix*, *Lactobacillus* and *Listeria*. Although facultatively anaerobic, *Brochothrix* spp. grow best aerobically. This feature, together with their ability to grow on unsupplemented nutrient agar, serves to distinguish *Brochothrix* spp. from members of the genera *Erysipelothrix* and *Lactobacillus* but not from *Carnobacterium* and *Listeria*.

The production of catalase distinguishes *Brochothrix* spp. from the genera *Carnobacterium*, *Erysipelothrix*, and the vast majority of *Lactobacillus* spp., but not from *Listeria*. But as noted previously, care must be taken in examining *Brochothrix* for catalase because its production depends on both the growth medium and the incubation temperature. Growth on APT medium, BAB no. 2 or a similar medium for 24–48 h at 20–25°C is recommended; negative results are very frequently obtained if the bacteria are incubated at 30°C. Although catalase production by *Listeria* spp. is also dependent on the composition of the growth medium (Jones, 1975), all strains of *Listeria* are unequivocally catalase-positive at 35°C if the growth medium is suitable, whereas *Brochothrix* spp. do not grow at this temperature.

The genera *Brochothrix* and *Listeria* share many common characters. Members of both genera are facultatively anaerobic but grow better aerobically; produce acid from a variety of sugars; produce catalase, cytochromes, and menaquinones; possess a cell wall peptidoglycan with *meso*-diaminopimelic acid as the diamino acid; have similar polar lipid and fatty acid profiles and similar G+C content (Table 1). Members of neither genus grow on acetate medium and only poorly on MRS (De Man et al., 1960) medium. In contrast to the comment by Jones (1992), *B. thermosphacta* and *B. campestris* do grow on MRS medium, though the type strain of the latter species grows more slowly. Members of *Listeria* and *Brochothrix* may be distinguished by their morphology, by growth temperature, by motility, and also serologically.

Table 2. Features most useful in differentiating *Brochothrix* from morphologically similar genera.

	Brochothrix	*Listeria*	*Carnobacterium*	*Lactobacillus*	*Erysipelothrix*	*Kurthia*
Growth at 35°C	−	+	+	+	+	+
Motility	−	+	−	−	−	+
Catalase	+	+	−	−	−	+
Facultatively anaerobic	+	+	+	+	+	−
Acid from glucose	+	+	+	+	+	−
H$_2$S production	−	−	−	−	+	−
Major peptidoglycan di-amino acid	*meso*-A$_2$pm	*meso*-A$_2$pm	*meso*-A$_2$pm	Lysine or *meso*-A$_2$pm or ornithine	L-Lysine	L-Lysine
Major menaquinone	MK-7	MK-7	−	−	−	MK-7
Major fatty acid types	S, A, I	S, A, I	S, U, (C)	S, U, (C)	S, A, I, U	S, A, I
G+C mol%	36–38	36–42	33–37	32–53	36–40	36–38

Symbols and abbreviations: +, present; , absent; MK-7, menaquinones with seven isoprene units; S, straight chain saturated; A, *anteiso*-methyl branched; I, *iso*-methyl-branched; U, monounsaturated; and (C), cyclopropane ring fatty acids may be present.

Colonies of the genus *Brochothrix* do not show the blue-green coloration exhibited by *Listeria* spp. when viewed by obliquely transmitted white light. The inability of *Brochothrix* spp. to grow at 35°C and their lack of motility also distinguishes them from the genus *Listeria*. No serological crossreactions have been demonstrated between *B. thermosphacta* and species of the genus *Listeria*.

General Properties

Metabolism and Nutritional Requirements

Brochothrix spp. are aerobic, oxidase negative, catalase positive and facultatively anaerobic (Sneath and Jones, 1986; Talon et al., 1988), but better growth is achieved by *B. thermosphacta* aerobically (see Hitchener et al., 1979 and Gardner, 1981); no such information is yet available for *B. campestris*.

Brochothrix thermosphacta possesses enzymes for both the hexose-monophosphate and Embden-Meyerhof (glycolysis) pathways of glucose catabolism as well as a number of enzymes involved in pyruvate metabolism (Collins-Thompson et al., 1972; Grau, 1983). Fermentative metabolism of glucose always results in the production of L(+) lactic acid, but other end products depend on the growth conditions. McLean and Sulzbacher (1953) found only L(+) lactic acid present in detectable quantities. Davidson et al. (1968b) detected small amounts of acetic and propionic acids in addition to L(+) lactic acid. Hitchener et al. (1979) found that in glucose-limited continuous culture under anaerobic conditions, L-lactate and ethanol were produced in the approximate ratio of $3:1$. The studies of Grau (1983) showed the major end products of anaerobic glucose fermentation to be primarily L-lactate, acetate, formate and ethanol, but that the ratios of these end products varied with conditions. Both the presence of acetate and formate and a pH below 6 increased L-lactate production from glucose. Of interest in this context is that the growth of *B. thermosphacta* is inhibited at a pH below 5 (Brownlie, 1966). Although McLean and Sulzbacher (1953) reported detectable CO_2 production (in Eldridge fermentation tubes), this has not been confirmed in any subsequent studies, but Hitchener et al. (1979) speculated that CO_2 was a likely end product in their studies on glucose-limited cultures.

The major end products of aerobic metabolism of glucose by *B. thermosphacta* growing on tryptone-based medium, on a minimal defined medium, or on meat are acetoin and acetic, isobutyric, isovaleric (3-methylbutyric), and 2-methylbutyric acids (Dainty and Hibbard, 1980; Dainty and Hibbard, 1983). In the minimal defined medium, glucose is believed to be the source of all the end products (Dainty and Hibbard, 1983); whereas in the complex medium and meat, only acetoin and acetic acid are derived from glucose; and isobutyric, isovaleric, and 2-methylbutyric acids are produced from valine, leucine and isoleucine, respectively (Dainty and Hibbard, 1980; Dainty and Hibbard, 1983). These compounds, or their derivatives, produce the sweet, sickly, malty odors that characterize the growth of *B. thermosphacta* (Dainty et al., 1985). The studies of Grau (1988) indicate that the substrates used by *B. thermosphacta* growing aerobically on meat include glucose, ribose, glycerol, glycerol-3-phosphate, and inosine; of these substrates, only glucose and ribose are metabolized during anaerobic growth.

In cultures of *B. thermosphacta* grown on a tryptone-based medium, enzymes of the tricarboxylic acid (TCA) cycle are almost totally absent (Collins-Thompson et al., 1972). However, Grau (1979) has suggested, on the basis of studies with a defined medium, that the TCA-cycle enzymes may be sufficiently active to provide substrates but not energy for synthesis.

Collins-Thompson et al. (1971) demonstrated the presence of a glycerol ester hydrolase (lipase) in cell suspensions and cell-free extracts of *B. thermosphacta*. The lack of significant lipase activity in supernatant fluids was confirmed by Papon and Talon (1988). This lipase, mostly produced during the logarithmic phase of growth is active on tripropionin, tricaproin, tricaprylin and trilaurin but not on tripalmitin. Because the temperature optimum of the lipase is 35–37°C with little or no activity below 20°C, this enzyme is unlikely to be important in meat stored at refrigeration temperatures; optimal temperatures for growth and lipase production was 24°C. The report that *B. thermosphacta* cultures attack tributyrin (Sutherland et al., 1975) has not been subsequently confirmed (Davis et al., 1969b; Patterson and Gibbs, 1978), and Papon and Talon (1988) could even demonstrate that growth and lipase production is inhibited by tributyrin.

Brochothrix thermosphacta requires cysteine, α-lipoate, nicotinate, pantothenate, *p*-aminobenzoate, biotin, and thiamine for growth (Grau, 1979). Thiamine can fulfil most, but not all, of the yeast extract requirements (Macaskie et al., 1981).

The production of cytochromes and catalase by *B. thermosphacta* depends on both the composition of the growth medium and the temperature of incubation (Davidson and Hartree, 1968a; Davidson et al., 1968b). The latter authors noted that *B. thermosphacta* strains grown on

APT medium (Baltimore Biological Laboratories [BBL], Cockeysville, MD; Evans and Niven, 1951) incubated at 20°C were always catalase-positive but that weak or negative reactions were obtained on HIA (heart infusion agar) medium (Difco, Detroit, MI) incubated at the same temperature The same authors reported that negative results were frequently obtained if the bacteria were grown on either medium at 30°C. Davidson and Hartree (1968a) reported the same effects of growth medium and incubation temperature on the quantitative cytochrome content of the organism. On APT medium incubated at 20°C, *B. thermosphacta* contains cytochromes baa_3 (Davidson and Hartree, 1968a). The temperature effect is difficult to explain; although not the optimum temperature for growth, *B. thermosphacta* grows well at 30°C. The effect of the composition of the culture medium may be related to the iron concentration. Davidson et al. (1968b) noted that APT medium contains added iron (8.0 µg/ml), and more recently both Grau (1979) and Thomson and Collins-Thompson (1986) have noted a high ferric iron requirement for the aerobic growth of *B. thermosphacta* in defined media. More recently it was shown that manganese can partially replace the iron requirement of *B. thermosphacta* under iron-limiting conditions (Thomson and Collins-Thompson, 1988).

Temperature and pH

There is general agreement that the temperature limits of growth are between 0 and 30°C (see Sneath and Jones [1976], Gardner [1981], and Talon et al. [1988]). Limited growth of *Brochothrix thermosphacta* was noted at 35°C and one strain was reported to grow at 37°C and 45°C (Gardner, 1981), but growth above 30°C has been rarely found. *Brochothrix campestris* does not grow at 37°C (Talon et al., 1988). The optimum temperature for growth is 20–25°C (see Gardner, 1981). The heat resistance of *B. thermosphacta* has received much attention because of its former classification in the genus *Microbacterium*, members of which are thermoduric. All workers agree that *B. thermosphacta* does not survive heating at 63°C for 5 min (see Gardner [1981] and Sneath and Jones [1986]).

The optimum pH for growth of *B. thermosphacta* is pH 7.0, but growth occurs within the range pH 5–9 (Brownlie, 1966).

Inhibitory Substances

The ability of *Brochothrix thermosphacta* to grow in the presence of NaCl has been examined by many workers (see Gardner, 1981). All strains grow at 6.5% NaCl and the majority can grow at NaCl concentrations up to 10% (Wilkinson and Jones, 1977; Talon et al., 1988). *Brochothrix campestris* strains do not grow in media with 8% or 10% NaCl and although there is no published information to indicate growth at 6.5% NaCl, circumstantial evidence indicates that *B. campestris* grows with 6.5% NaCl.

Growth of *B. thermosphacta* is inhibited by nitrite, but the degree of inhibition is related to the pH of the medium and incubation temperature (Brownlie, 1966); low pH, low temperature, and high nitrite increase the inhibitory effect. The species does not appear to have a nitrite reductase system (Collins-Thompson and Rodriguez-Lopez, 1980). Dainty and Meredith (1972) found that 200 parts per million (ppm) of nitrite at pH 5.5 stops RNA, DNA, and protein synthesis in *B. thermosphacta* but has no effect on membrane permeability. An interesting study on the combined effects of NaCl, $NaNO_2$, temperature, and pH on the growth of *B. thermosphacta* in broth cultures was done by Roberts et al. (1979). Also, sodium lactate has been used effectively against *B. thermosphacta* (reduction by 4 logs as compared to the control after 14 days; Lemay et al., 2002). Microgard 100, Microgard 300, Alta 2002, and Perlac 1902 had no significant effect on tests using an acidified chicken meat model.

In the United Kingdom, sulfur dioxide is permitted in sausage meat and *B. thermosphacta* tolerates SO_2 up to 500 ppm under both aerobic and anaerobic conditions (Dowdell and Board, 1971).

The studies of Macaskie (1982) indicate that in liquid culture palmitic acid is inhibitory to the growth of *B. thermosphacta*.

In plate overlay assays, triclosan-incorporated plastic (TIP) inhibited, among other Gram-positive and Gram-negative organisms, also *Brochothrix thermosphacta*. When TIP was used to cover irradiated, lean beef surfaces inoculated with various bacteria, only populations of *B. thermosphacta* were reduced (Cutter, 1999).

Brochothrix thermosphacta became the main species responsible for putrefaction when poultry carcasses were decontaminated with AvGuard (or Assur-Rinse in the United States) trisodium phosphate. This treatment preferentially removed pseudomonads with the consequence that *B. thermosphacta* lacked competition for utilization of substrates; as however, growth rate of *B. thermosphacta* was greater than that of *Listeria monocytogenes*, putrefaction would occur before the emergence of large numbers of the latter species (Salvat et al., 1997).

Brochothrix thermosphacta is more resistant to irradiation than common meat spoilage organisms such as *Pseudomonas* (Ouattara et al., 2002)

but are affected by irradiation doses of 0.5 and 2.0 kilogray (kGy; Savvaidis et al., 2002). The species has been frequently isolated from irradiated meat and poultry (see Gardner, 1981).

Serology

There do not appear to have been any systematic serological studies of *Brochothrix* spp. Wilkinson and Jones (1975) did not detect any serological relationship between *B. thermosphacta* and the genera *Erysipelothrix*, *Kurthia* or *Listeria* with antisera raised against representative strains of all four taxa.

Esterase Isoenzymes

Five different esterase isoenzymes have been detected by gel electrophoresis studies of *Brochothrix thermosphacta*. Different combinations of these enzymes resulted in the detection of seven groups among 26 strains examined. There was no correlation between source of isolation and groups based on esterase patterns (G. A. Gardner, personal communication).

Bacteriophages

Greer (1983) isolated bacteriophages active on *Brochothrix thermosphacta* from aqueous extracts of spoiled beef. Both phage plaque size and plating efficiency increased significantly when the incubation temperature was reduced from 25°C to 1°C. The detection of 14 distinct phage lysotypes led Greer (1983) to suggest that phage typing may provide a rapid method of differentiating *B. thermosphacta* strains.

On the basis of their morphology as determined by electron microscopy, Ackermann et al. (1988) grouped these bacteriophages (and two isolated in France from broth cultures of lysogenic *B. thermosphacta* strains) into three viral species of the Myoviridae (species A19) or Siphoviridae (species NF5 and BL3). The bacteriophage species A19 are interesting because of their similarity to some bacteriophage species of the genera *Bacillus*, *Lactobacillus*, *Staphylococcus* and *Streptococcus*. Surprisingly, none of the bacteriophages resembled those of the genus *Listeria* (Ackermann et al., 1988).

Bacteriophages have been used to control *B. thermosphacta* spoilage of pork adipose tissue. In the presence of 10^5 bacteria per cm^2 tissue disk and an equivalent number of phages, phage numbers increase by 1000-fold while bacterial number decreased by a factor of 100. As a result, off-odors were suppressed during refrigeration, allowing increase of the storage life of the adipose tissue (Greer and Dilts, 2002).

Plasmids

Dodd and Waites (1988) detected plasmids in all strains of *Brochothrix thermosphacta* isolated from sausages containing 450 ppm of sulfite, but not in the type strain (NCTC [{www.ncbi.nlm.nih.gov /entrez/query.fcgi?db=nucleotide =search=10082}{10082}]). The plasmids ranged in size from 1600 to 43,000 and plasmid numbers varied from 1 to 8 per cell. Comparison of the plasmid profiles of the isolates showed that fewer profiles were present amongst the strains isolated from sausages stored for various periods at 4°C than were present amongst strains isolated from the same sausages on the day of manufacture. Dodd and Waites (1988) interpreted these observations as indicating that only some strains of *B. thermosphacta* were able to survive the conditions of storage.

Bacteriocins

Brochocin C, produced by *Brochothrix campestris*, was discovered and classified (Siragusa and Cutter, 1993) as a bacteriocin active against *B. thermosphacta*, *Listeria monocytogenes* and several strains of *Carnobacterium*, *Kurthia*, *Lactobacillus*, *Pediococcus*, *Streptococcus* and even endospores of *Clostridium* and *Bacillus* spp. (McCormick et al., 1998). In its activity spectrum, brochocin C is similar to the lantibiotic nisin A. Brochocin C is a nonlantibiotic class II bacteriocin, consisting of two peptides (BrcA and BrcB) that are the result of a Gly-Gly cleavage of a prepeptide. Both peptides are necessary for activity (Garneau et al., 2003). Immunity against the bacteriocin is due to an immunity protein, encoded downstream of the *brcB* gene. In Gram-negative bacteria, the outer membrane acts as a barrier against nisin and brochocin (Gao et al., 1999).

Brochothrix spp, like other Gram-positive relatives, are inhibited by bacteriocins such as the thermosensitive peptide produced by *Streptococcus thermophilus* 580 (Mathot et al., 2003), salivaricin B, excreted by *Lactobacillus salivarius* M7, acidocin B from *Lactobacillus acidophilus* M46 (Ten Brink et al., 1994; Leer et al., 1995), and nisin. Nisin is widely studied as an agent controlling the growth of meat spoilage bacteria (Gill and Holley, 2000; Nattress et al., 2001; Nattress and Baker, 2003). As compared to free nisin, calcium-alginate-bound nisin as well as the meat binding system Fibrimex® plus nisin are more effective inhibitors of *B. thermosphacta* (Cutter and Siragusa, 1996; Cutter and Siragusa, 1998). Nisin-incorporated polymers (polyethylene and polyethylene oxide) also reduce *B. thermosphacta* in experimentally surface-inoculated vacuum packed beef (Cutter et al., 2001).

Habitats

The natural habitat of *Brochothrix* spp. is not known with certainty. This is mainly because for many years after the first reported isolation of the type species, *B. thermosphacta*, from finished pork sausage and its subsequent incrimination as an important psychrotrophic spoilage organism of meat and meat products, most investigators concentrated only on these materials (see Gardner [1981] and Keddie and Jones [1981]). During studies on the development of a selective medium for the enumeration of *B. thermosphacta* from meat sources, Gardner (1966) isolated the organism from soil and feces, but in low numbers. More recently *B. thermosphacta* has been isolated from a variety of sources that include fish and fish products, frozen foods, milk, and cream (see Gardner [1981] and Sneath and Jones [1986]). Both *B. thermosphacta* and *B. campestris* have also been isolated from soil and grass (Talon et al., 1988). To date, soil and grass are the only described habitats of *B. campestris*, but it is possible that this species has been misidentified as *B. thermosphacta* in the past. From the information available it seems probable that *Brochothrix* spp. are widely distributed in the environment and become a prominent part of the flora in habitats that selectively favor their growth.

The turbidimetric method of counting *Brochothrix thermosphacta* correlated well with bacterial numbers determined by plate counts, flow cytometry, and manual counts (by microscope) over a limited range of 10^7–10^9 cells/ml. Flow cytometry and manual counts gave a linear relationship over a wider range of 10^5–10^9 cells/ml. Passage of *B. thermosphacta* cells through the flow cytometer resulted in the breakage of chains into single cells which makes this technique attractive for bacterial enumeration (Rattanasomboon et al., 1999).

Meat and Meat Products

Since *Brochothrix thermosphacta* was first isolated from pork trimmings and finished sausage (Sulzbacher and McLean, 1951), it has been isolated regularly from the same sources and from a variety of meats (including poultry) and meat products (see the detailed list of references in Jones, 1991). An excellent, comprehensive review of the isolation of *B. thermosphacta* from, and its ecological association with, beef, lamb and pork, and cured and uncured products made from these meats and poultry meat was done by Gardner (1981). Most of the recent reports deal with verification of *B. thermosphacta* in meat subjected to different preservation regimes, e.g.,

Gill and Bryant (1992), Prieto et al. (1993), Kakouri and Nychas (1994), Borch et al. (1996), Jimenez et al. (1997), Lopez-Caballero et al. (1999), Samelis and Georgiadou (2000), Silv et al. (2000), Gill and Badoni (2002), Gonzalez-Rodriguez et al. (2002), Bohaychuk and Greer (2003), Susiluoto et al. (2003), and Greer et al. (2004).

Brochothrix thermosphacta is especially important as a spoilage organism in prepacked meats and meat products stored at chill temperatures (see Gardner, 1981). The conditions prevailing during such storage selectively favor its growth (Gardner et al., 1967). *Brochothrix* spp. grow at temperatures as low as 0°C and under conditions of O_2 depletion and increased CO_2 concentration (Pin et al., 2002). Other factors that inhibit or enhance the growth of *B. thermosphacta* during the storage of particular meats (including poultry) and meat products are reviewed by Gardner (1981). These include the chemical and physical properties of the various meats; temperature of storage; composition of gaseous environment; pH; presence of NaCl, $NaNO_2$ or SO_2; previous heat treatment; and interactions with other spoilage bacteria (see also Nychas et al., 1988).

Unlike proteolytic spoilage bacteria (e.g., *Pseudomonas*), *B. thermosphacta* is usually found only on the meat surface (Gill and Penney, 1977), and in prepacked meats, it grows well at the meat-plastic film interface (Ingram and Dainty, 1971). However, Fournaud et al. (1980) demonstrated histologically that meat spoilage bacteria, including *B. thermosphacta*, can penetrate the deep muscle mass by following the perimysium or endomysium connective tissue. Of interest is their observation that the flora of deep muscle tissue stored aerobically was dominated by *B. thermosphacta* whereas that of vacuum-packed meat was dominated by *Lactobacillus*.

Contamination of meats almost certainly occurs during slaughter and postslaughter procedures. *Brochothrix thermosphacta* has been isolated from the hides of cattle (Mulder, 1978; Newton et al., 1978); cattle hair, rumen contents, floors and equipment in slaughter halls (Patterson and Gibbs, 1978; Talon et al., 1988); sheep wool and sheep feces (Talon et al., 1988); equipment and tables used in the preparation of sausage (McLean and Sulzbacher, 1953); and cooked rind and fat emulsions added to pork sausages (Gardner, 1981).

Fish and Fish Products

In the last 10 years, *Brochothrix thermosphacta* has been isolated from fish sources, but it does not appear to be as economically important; nor does it comprise as large a proportion of the

microflora as it does in meat. Nickelson et al. (1980) isolated *B. thermosphacta* at all stages in the production (whole fish, scaled, beheaded, eviscerated, and minced flesh) of minced fish flesh from nontraditional finfish caught in the Gulf of Mexico, but the proportion of the total microflora rarely exceeded 5%. Lannelongue et al. (1982) isolated *B. thermosphacta* from stored finfish fillets packaged in a CO_2 atmosphere. Again the numbers recovered as a proportion of the total microbial population were small. The species has also been isolated from fish fingers, smoked whiting, frozen coley, and frozen cod (Gardner, 1981). Little information on *Brochothrix* detected on fish has been published in the past 10 years. Investigation of vacuum-packed cold-smoked salmon and trout has indicated a low number of *Brochothrix* spp. (Gonzalez-Rodriguez et al., 2002). These organisms have also been detected in the Mediterranean boque (*Boops boops*) but were found to constitute a population with a density of 2–3 $\log_{10}$ CFU g^{-1}, less than that of pseudomonads (Koutsoumanis and Nychas, 1999).

Brochothrix campestris has not been observed in fish or fish products but prior to the study of Talon et al. (1988), misidentification could have occurred. Talon et al. (1988) did not examine these sources.

Other Sources

Brochothrix thermosphacta has been isolated from soil, grass, hay, and feces but apparently only in low numbers and with the use of selective media. There have also been reports of the isolation of the species from a variety of foods such as frozen peas, frozen runner beans, prepackaged tomato salad, milk, cream, and cottage cheese (Gardner, 1981).

As noted earlier, the only currently known habitats of *B. campestris* are grass and soil (Talon et al., 1988). Inspection of the numbers of isolates of *B. thermosphacta* and *B. campestris* from grass and soil studied by these workers does not indicate that *B. campestris* forms a larger proportion of the microflora of these habitats than does *B. thermosphacta*; no other indication of the relative proportions of the two species in grass and soil samples is reported (Talon et al., 1988).

Neither *B. thermosphacta* nor *B. campestris* appear to have been isolated from clinical sources.

Isolation of Brochothrix

Until recently the methods described for the isolation of *Brochothrix* have been mainly concerned with the isolation of *B. thermosphacta*

from meats and meat products. *Brochothrix campestris* grows equally well on the same media. Neither species grows on the acetate medium devised by Rogosa et al. (1951) for the isolation of lactobacilli (see Keddie and Jones [1981] and Talon et al. [1988]). *Brochothrix thermosphacta* (no information is available for *B. campestris*) grows only poorly on the MRS medium of (De Man et al., 1960). Several different media based on peptone, yeast extract, and glucose or glycerol have been used for the isolation of *B. thermosphacta* (Sulzbacher and McLean, 1951; Wolin et al., 1957; Barlow and Kitchell, 1966; Gardner, 1966; Gardner, 1967). More recently, the streptomycin sulfate-thallous acetate-actidione (actidione = cycloheximide) agar medium (STAA) of Gardner (1966) has been used routinely for the selective isolation and enumeration of *B. thermosphacta* from meats, meat products, fish and a variety of other foods (Gardner, 1981). Although extremely useful for such materials, the selectivity of STAA is poor when soils or fecal samples are examined (Gardner, 1981). Talon et al. (1988) used STAA medium supplemented with nalidixic acid and oxacillin for the isolation of *B. thermosphacta* from soil, grass, feces, etc., and for the isolation of *B. campestris* from soil and grass.

Isolation of *Brochothrix thermosphacta* from Meats and Meat Products.

Swabs of various meat surfaces or samples of macerated meat or other materials are usually suspended in 0.1% (w/v) peptone water and shaken vigorously before plating on suitable media (see Gardner et al., 1967). Plates should be incubated at 22°C for up to 5 days, but colonies of *B. thermosphacta* are usually visible within 48 h.

The following media are suitable for the growth of *B. thermosphacta*.

Glycerol Nutrient Agar (Gardner, 1966; Gardner, 1967)

Peptone (Oxoid)	20 g
Yeast extract (Oxoid)	2 g
Glycerol	15 g
K_2HPO_4	1 g
$MgSO_4 \cdot 7H_2O$	1 g
Agar (Oxoid no. 3)	13 g

Dissolve ingredients and dilute up to 1 liter with distilled water. Adjust pH to 7.0 and autoclave at 121°C for 15 min.

Glucose Nutrient Agar (Sulzbacher and McLean, 1951; Wolin et al., 1957)

Tryptone (Difco)	10 g
Yeast extract (Difco)	5 g
K_2HPO_4	5 g
NaCl	5 g
Glucose	5 g
Agar	15 g

Dissolve ingredients and dilute up to 1 liter with distilled water. Adjust pH to 7.0 and autoclave at 121°C for 15 min.

Medium STAA for Selective Isolation of *Brochothrix thermosphacta* (Gardner, 1966)

Peptone (Oxoid)	20 g
Yeast extract (Oxoid)	2 g
Glycerol	15 g
K_2HPO_4	1 g
$MgSO_4 \cdot 7H_2O$	1 g
Agar (Oxoid no. 3)	13 g

Dissolve ingredients and dilute up to 1 liter with distilled water. Adjust pH to 7.0 and autoclave at 121°C for 15 min. To this sterile, molten medium, add the following solutions (prepared in sterile, distilled water) to give the final concentrations indicated; streptomycin sulfate (Glaxo), 500 µg/ml; cycloheximide (Upjohn), 50 µg/ml; and thallous acetate, 50 µg/ml).

After incubation of appropriate samples on this medium at 20–22°C for 48 h, a large majority of the colonies are *B. thermosphacta*; the exceptions are a few pseudomonads.

Modified Medium STAA for Selective Isolation of *Brochothrix* spp. from Grass, Soil, Feces, and Similar Material (Talon et al., 1988)

Prepare as for STAA medium but supplement with nalidixic acid (15 µg/ml) and oxacillin (5 µg/ml).

After incubation of appropriate samples on this medium at 20–22°C for 48 h, a large majority of the colonies are *Brochothrix* spp.

Enrichment of *Brochothrix* spp.

Enrichment of *Brochothrix* spp. is not usually performed, but holding meat or meat product samples under gas-permeable film at temperatures below 10°C can act as enrichment. Similar enrichment for other materials, grass or soil, could be useful but apparently has not been attempted.

Preservation of Cultures

Cultures may be preserved for short periods (6 months to 1 year) in nutrient agar (plus 0.1% glucose) stabs in screw-capped bottles stored in the dark at room or refrigeration temperature. Longer-term preservation (over 10 years with *Brochothrix thermosphacta*) may be achieved by freezing on glass beads at –60 to –70°C (Jones et al., 1984). Cultures may also be preserved by freeze drying (lyophilization).

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CHAPTER 1.2.13

The Genus *Erysipelothrix*

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The genus *Erysipelothrix* consists of three species, *Erysipelothrix rhusiopathiae* (Rosenbach, 1909), *Erysipelothrix tonsillarum* (Takahashi et al., 1987a) and the recently described *Erysipelothrix inopinata* (Verbarg et al., 2004). In 1876, Koch first isolated this slender, pleomorphic, Gram-positive bacillus from the blood of mice that had been inoculated subcutaneously with blood from putrefied meat (Koch, 1878) and was designated "*E. muriseptica*." In 1882, Loeffler observed a similar organism in the cutaneous blood vessels of a pig that had died of swine erysipelas and published the first good description of the organism (Loeffler, 1886). It is probable that a bacillus observed a few months previously by Pasteur and Dumas in pigs dying of rouget (swine erysipelas) was the same organism as that described by Loeffler (Pasteur and Dumas, 1882). Trevisan (1885) proposed the name "*E. insidiosa*." Rosenbach was the first to establish *Erysipelothrix* as a human pathogen. In 1909, he reported the isolation of the organism from a patient with localized cutaneous lesions and coined the term "erysipeloid" to distinguish these lesions from those of human erysipelas (Rosenbach, 1909). Subsequently, *Erysipelothrix* was identified as the cause of infection in many animal species.

Rosenbach distinguished three species, *E. muriseptica*, *E. porci* and *E. erysipeloides*, based on their murine, porcine, and human origins, respectively (Rosenbach, 1909). The name *Bacterium rhusiopathiae* (Migula, 1900) antedated the name *E. porci*. The combination *Erysipelothrix rhusiopathiae* was first proposed by Buchanan (1918). At least 36 names have appeared in the literature for species of this genus. With the appreciation that all strains belonged to a single species, the name *E. insidiosa* was proposed for *E. rhusiopathiae*, *E. muriseptica*, and *E. erysipeloides* (Langford and Hansen, 1953; Langford and Hansen, 1954). In 1966, Shuman and Wellmann proposed that the name *E. insidiosa* be rejected in favor of *E. rhusiopathiae* which means literally "erysipelas thread of red disease."

With more strains being subjected to taxonomic studies, the great variation in serological, biochemical, chemical and genomic properties of *E. rhusiopathiae* was noted (Erler, 1972; Feist, 1972; Flossmann and Erler, 1972; White and Miritikani, 1976; Takahashi et al., 1992) and consequently the species *E. tonsillarum* (also named "*E. tonsillae*" in the older literature [Takahashi et al., 1989] was described for avirulent *Erysipelothrix* strains of serotype 7, frequently isolated from the tonsils of apparently healthy pigs (Takahashi et al., 1987a). The species *E. inopinata* was isolated from sterile-filtered vegetable broth (Verbarg et al., 2004).

Erysipelothrix, the only genus of the family Erysipelotrichaceae (Verbarg et al., 2004), is defined as Gram-positive but may appear Gram-negative because it decolorizes readily. Strains are nonencapsulated, nonsporulating, nonmotile straight, or slightly curved, slender rods with rounded ends, but some show a tendency to form long filaments. Organisms are arranged singly, in short chains, or in pairs in a "V" configuration or are grouped randomly. They are not acid-fast. Menaquinones (MKs) are absent. The exact growth requirements of the organism have not been determined. Several amino acids, riboflavin, and small amounts of oleic acid are required (Hunter, 1942), and growth is enhanced by tryptophan (Ewald, 1981). Growth occurs at 15–44°C, with an optimal temperature of 30–37°C, and at a pH of 7.2–7.6 with a range of 6.7–9.2 (Sneath et al., 1951). The organism is a facultative anaerobe. Growth is improved by 5–10% carbon dioxide. Heating at 60°C for 15 min is lethal. *Erysipelothrix rhusiopathiae* is able to grow in the presence of 0.2% phenol, 0.1% sodium azide, 0.001% crystal violet, 0.05% potassium tellurite, 0.02% thallous acetate, and 0.2% 2,3,5, triphenyl-tetrazolium chloride (Sneath et al., 1951; Ewald, 1981). Cells are catalase-negative and oxidase negative. The cell wall is Gram positive; the murein belongs to the B-crosslinking type, having L-alanine in position three of the peptide subunit and an interpeptide bridge consisting of the peptide Gly→L-Lys→L-Lys. 16:0, *cis*-9-18:1 and 18:0 are the predominant fatty acids. Cells are aerobic to facultatively anaerobic, chemoorganotrophic with respiratory metabolism, and weakly fermentative. Acid but no gas produced from glucose and other carbo-

hydrates. The mol% of the DNA G+C is 36–40% (high pressure liquid chromatography [HPLC], melting temperature [Tm], and buoyant density [Bd]; Flossmann and Erler, 1972). Some strains pathogenic for mammals and birds.

Review-type coverage of the biology and clinical features of *Erysipelothrix* has been dealt with by Takahashi (Takahashi, 1996; in Japanese), Hill and Ghassemian (1997), Brooke and Riley (1999), Brouqui and Raoult (2001), and Boo et al. (2003), and in The Genus Erysipelothrix in the second edition.

Taxonomy and Phylogeny

Erysipelothrix was once thought to be closely related to *Listeria* (Barber, 1939), but results of studies of cell wall peptidoglycan (Schleifer and Kandler, 1972; Verbarg et al., 2004), fatty acid patterns (Tadayon and Carroll, 1971), DNA hybridization studies (Stuart and Welshimer, 1974), and numerical taxonomic studies (Davis and Newton, 1969; Stuart and Pease, 1972b; Jones, 1975; Wilkinson and Jones, 1977; Feresu and Jones, 1988) did not support this relationship. There are no common antigens between strains of *Erysipelothrix* and *Listeria monocytogenes* as detected by immunodiffusion or passive hemagglutination tests (Pleszczynska, 1972a; Pleszczynska, 1972b). Differences between the two genera have been demonstrated in cell wall chemistry by chromatography and infrared spectrophotometry. Paper and thin-layer chromatography (TLC) of acid hydrolysates of the purified cell wall show that *Erysipelothrix* is clearly distinguishable from *Listeria*. While members of *Erysipelothrix* have lysine and glycine in the cell wall (Mann, 1969), *Listeria* possesses *meso*-diaminopimelic acid.

In the 1980s, the genus *Erysipelothrix* was classified as a regular nonsporeforming Gram-positive rod (Jones, 1986). However, even earlier, enzyme and DNA-base ratio studies reveal a closer relationship of *Erysipelothrix* to the family Lactobacillaceae than to Corynebacteriaceae (Flossmann and Erler, 1972; White and Mirikitani, 1976). In a study of more than 200 strains of coryneform bacteria using 173 morphological, physiological, and biochemical tests and computer analysis, eight clusters were identified, and *Erysipelothrix* was most closely related to *Streptococcus pyogenes* (Jones, 1975). In another study, the closest similarity of *Erysipelothrix* was to the genus *Gemella* (Wilkinson and Jones, 1977). In general, results of numerical taxonomic studies indicate that strains of *E. rhusiopathiae* form a distinct cluster that shows the closest similarity to the streptococci (Jones, 1986).

With the introduction of 16S rRNA gene sequence analyses, the phylogenetic position has been elucidated and today the genus is considered a member of the Firmicutes (Kiuchi et al., 2000; Verbarg et al., 2004) belonging to the family Erysipelothricaceae (*Clostridium* cluster XVI according to Collins et al., 1994, and Verbarg et al., 2004), a lineage that also embraces *Holdemania filiformis* (Willems et al., 1997). Less closely related are *Bulleidia extructa* (Downes et al., 2000), *Solobacterium moorei* (Kageyama et al., 2000), and nonauthentic members of *Eubacterium*, *Streptococcus* and *Clostridium* (see Fig. 1). The 16S rRNA gene sequences of the *E. rhusiopathiae* strain [{genbank}{AB055905}] is highly similar to the sequence of *E. tonsillarum* [{genbank}{AB055906}] (99.8% similarity). DNA-DNA reassociation values obtained for these strains range only between 18 and 36%, which confirm differences at the physiological level, hence their separate species status. The 16S
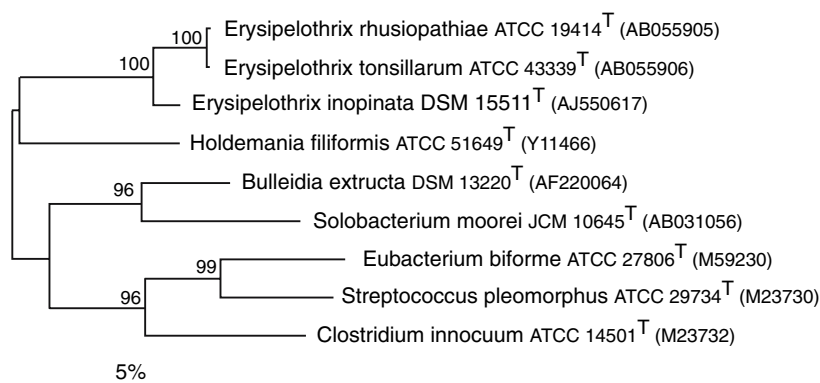
Fig. 1. 16S rRNA gene sequence dendrogram (De Soete, 1983) displaying the phylogenetic position *Erysipelothrix* strains among some phylogenetic neighbors. Similarity values obtained for almost complete sequences were transformed into phylogenetic distance values that compensate for multiple substitutions at any given site in the sequence (Jukes and Cantor, 1969). Numbers indicate the percentage of bootstrap (> 95%) samplings (Felsenstein, 1993), derived from 1000 resamplings. Bar indicates sequence divergence. Numbers in parentheses are accession numbers of 16S rRNA gene sequences.

rRNA gene sequence of *E. inopinata* [{gen-bank}{AJ550617}] is less than 97.5% similar to that of the other two type strains, while it shares 99.9% similarity to that of strain Pecs 56 ([{gen-bank}{AB055907}]) listed as unpublished in the European Molecular Biology Laboratory (EMBL) database. 16S rDNA gene sequences of *E. rhusiopathiae* strains serotype 13 ([{gen-bank}{AB019249}]) and serotype 18 ([{gen-bank}{AB019250}]; Takeshi et al. 1999), covering the 3' half of the molecule (about 790 nucle-otides), share 97.5 and 97.8% similarity, re-spectively, with the corresponding fragment of the gene of strain DSM 15511^T [{gen-bank}{AB019250}].

Habitats

Occurrence in Animals

Erysipelothrix rhusiopathiae and infections due to this organism occur worldwide. It has been found as a commensal or a pathogen in a wide variety of vertebrate and invertebrate species, including "swine, sheep, cattle, horses, dogs, wild bears, kangaroos, reindeer, mice, wild rodents, seals, sea lions, cetaceans, mink, chipmunks, crus-taceans, fresh and salt-water fish, crocodiles, caimans, stable flies, house flies, ticks, mites, mouse lice, turkeys, chickens, ducks, geese, guinea fowl, pigeons, sparrows, starlings, eagles, parrots, pheasants, peacocks, quail, parakeets, mud hens, canaries, finches, siskins, thrushes, blackbirds, turtledoves, and white storks" (Conklin and Steele, 1979; see also Creech, 1921; Gledhill, 1948; Woodbine, 1950; Sneath et al., 1951, Hunter, 1974, and Wood, 1975). Recent reports verify the occurrence in chickens (Mohan et al., 2002), chicken meat (Nakazawa et al., 1998), dogs (Takahashi et al., 1993; Taka-hashi et al., 2000), wild boars (Yamamoto et al., 1999), moose (Campbell et al., 1994), opossum (Lonigro and LaRegina, 1988), and retail pork and fish (Stenstrom et al., 1992). Some of the reports on *E. rhusiopathiae* may actually cover strains of *E. tonsillarum*. The major reservoir of *E. rhusiopathiae* is generally believed to be domestic swine, but rodents and birds are also frequently infected. The organism causes no known disease in fish but can grow and persist for long periods in the mucoid exterior slime of these animals (Wood, 1975).

The presence of *E. rhusiopathiae* in the external environment is probably secondary to contamination by infected animals; it may be found in sewage effluent from abattoirs and on ground contaminated with the feces of animals with apparent or inapparent infection. It now appears that, contrary to previous belief, the organism is not able to exist indefinitely in soil, but it may live long enough to cause infection in animals or humans weeks or months after initial soil contamination. Pathogenic strains of *E. rhusiopathiae* have been isolated from the feces of apparently healthy swine, and asymp-tomatic swine commonly harbor this organism in their tonsils and other lymphoid tissues (Wood, 1974a). Some of these isolates are included in *E. tonsillarum* (Takahashi et al., 1987a). Isolates of the genus have also been recovered from the tonsils of healthy cattle in Japan (Hassanein et al., 2001). A cycle may thus occur in which asymptomatic carrier swine contaminate the soil of the swine pens, and organisms in the soil are transmitted to previ-ously uninfected animals, some of which become asymptomatic carriers of these bacte-rial strains.

Occurrence in Humans

The risk of human infection with *E. rhusio-pathiae* is closely related to the opportunity for exposure to the organism (McGinnes and Spindle, 1934). Relation to age, sex, race, and socioeconomic status appears to reflect only opportunity for exposure. Most human cases are related to occupational exposure. Individu-als at greatest risk for infection include butch-ers, fishermen, fish handlers, abattoir workers, veterinarians and housewives (Klauder, 1938; Morrill, 1939; Woodbine, 1950; Hillenbrand, 1953; Hunter, 1974; Wood, 1975; Conklin and Steele, 1979; Gorby and Peacock, 1988), but erysipelothrix infection has been associated with a wide variety of occupations including "butchers, meat cutters, meat-processing work-ers, poultry-processing workers, meat inspec-tors, rendering plant workers, knackers, animal caretakers, farmers, fishermen, including lobster fishermen, fish and lobster handlers, fish-processing workers, crab and crayfish-processing workers, clam openers, veterinarians, including veterinary students, cooks, bakers, housewives, kitchen workers, food handlers, caterers, button makers (bone), game handlers, furriers, leather workers, soap makers, fertilizer workers, sewer workers, bacteriologists, laboratory workers, and stockyard workers" (Wood, 1975). Infec-tion is especially common among individuals who handle fish. Hunter (1974) noted the occurrence of "fish-handlers'" disease among "fishermen, fish cleaners, gutters and picklers, fish porters, fish-box repairers, fish-lorry drivers, fish-meal workers, smoke driers, fishmongers, cooks and housewives who infect themselves through abrasions of the skin caused by the spines, fins, and bones of fish, especially skate." During World War II, outbreaks of the disease

occurred in factories in Norway where fish was dried and tinned and cod heads made into fertilizer; the delay caused by the need of fishing boats to sail in convoy apparently allowed the organisms to multiply, resulting in the high incidence of infections (Hunter, 1974). Diseases called "seal finger" and "whale finger" occur in those who capture these animals and scratch their hands on the steel ropes used in their work (Hillenbrand, 1953). Anglers may be infected through puncture wounds made by fish hooks or the teeth of fish, or by the claws of lobsters and crabs; in the series of 329 cases of erysipeloid described by Gilchrist, 323 were caused by injuries produced by crabs (Gilchrist, 1904). Abattoir workers, meat porters, butchers and poulterers may become infected through small cuts from the knives they use or through abrasions caused by splinters of bone (a condition called "pork finger"). Veterinary pathologists may become accidentally inoculated by injury from knives or bone splinters during necropsy of infected animals, particularly poultry. Workers who peel potatoes and other root vegetables may become infected by earth contaminated with the manure of infected animals.

Most cases in humans and other animals probably occur via scratches or puncture wounds of the skin, but in some cases it appears that the organism has penetrated intact skin (McGinnes and Spindle, 1934). Human-to-human infection has not been documented. Although *E. rhusiopathiae* is killed by moist heat at 55°C for 15 minutes, it is resistant to many environmental influences, including salting, pickling, and smoking (Conklin and Steele, 1979). Meat and bacon may contain the organism after pickling for 170 days or after 30 days in a mixture of salt and potassium nitrate, and the organism has been recovered from ham after smoking. It may remain alive for 12 days in direct sunlight, and for many months in carcasses left to decay on the surface of the ground or buried as deep as 7 feet. It has also been found in city sewage containing drainage from abattoirs and stables.

Reporting of infections due to *E. rhusiopathiae* is not required by health authorities, so it is difficult to know whether the incidence of these infections is increasing or decreasing. Some technological changes in industries employing animal products have probably resulted in reduced contact between *E. rhusiopathiae* and humans. For example, nearly all buttons are now made of plastic, rather than bone. To the extent that such changes reduce occupational exposure to the organism, the future incidence of erysipeloid, and more serious forms of infection with *E. rhusiopathiae*, will decline.

Diseases Due to Erysipelothrix rhusiopathiae

Disease in Animals

Swine erysipelas is worldwide in distribution. It was first recognized as an important disease in the United States during the 1930s, and by 1959 it had been reported from 44 states. The incidence then declined to a relatively low level by 1972, where it has remained. The present low prevalence of swine erysipelas, in spite of the widespread distribution of the organism, may be attributible largely to management practices such as maintenance of closed herds, use of confinement housing, improved waste disposal, vaccination, and use of antibiotics in feed (Wood, 1984).

Four clinical entities have been described in swine: 1) an acute septic form, in which the animals may die within a few days; 2) a subacute urticarial form marked by reddish purple rhomboid spots or "diamonds" in the skin (diamond skin disease); 3) a joint or arthritic form; and 4) a chronic cardiac form (endocarditis; Klauder, 1944; Gledhill, 1948; Woodbine, 1950; Wood and Shuman, 1974b).

In sheep, the typical form of the disease is polyarthritis, which begins when the lambs are 2–3 months old (Conklin and Steele, 1979). The gait becomes stiff, and the animals have difficulty rising and may fall down. The involved joints are usually swollen and the capsule is thickened. The organism can be cultured from the joint tissue. There are no lesions in visceral organs.

Erysipelothrix rhusiopathiae causes disease in many species of birds (Conklin and Steele, 1979). Adult male turkeys develop a disease called "bluecomb" because of the cyanotic skin color. The birds become droopy, develop diarrhea, and die. Hemorrhagic lesions are found in the breast and leg muscles, pericardium, liver and spleen, and the organism is easily isolated from these tissues. Large outbreaks have also occurred in domestic ducks, less commonly in chickens.

Although the greatest commercial impact of *E. rhusiopathiae* infection is due to disease in swine, infection of sheep, turkeys, and ducks is also of economic importance (Conklin and Steele, 1979). Both killed bacterin vaccines (formalin-killed whole culture adsorbed on aluminum hydroxide gel) and live avirulent vaccines have been available in the United States since the 1950s. Both are prepared from organisms of serovar 2 but provide cross-protection against many (but not all) other serovars (Sawada and Takahashi, 1987). Effectiveness of killed and live vaccines appears to be approximately equal (Schuman, 1959). Usage in the United States has

roughly paralleled the incidence of swine erysipelas. Killed bacterins have also been used in turkeys, primarily during epizootics. An effective live avirulent vaccine that can be administered in drinking water has also been developed recently (Bricker and Saif, 1988).

Disease in Humans

The spectrum of disease seen in humans closely parallels that seen in swine (Klauder, 1926; Sikes, 1958; Grieco and Sheldon, 1970; Phillips, 1986). There are three well-defined clinical categories of human disease: 1) a localized cutaneous form, erysipeloid; 2) a generalized cutaneous form; and 3) a septicemic form, which is often associated with endocarditis (Grieco and Sheldon, 1970).

LOCALIZED CUTANEOUS FORM OR ERYSIPELOID OF ROSENBACH Erysipeloid is a localized skin infection that is actually a cellulitis. Because of its mode of acquisition, contact with infected animals, fish, or their products, with organisms gaining entrance via cuts or abrasions on the skin, lesions are usually confined to the fingers and hands. The patient complains of pain and swelling of the finger or part of the hand. The pain is often severe and may be described as a burning, throbbing or itching. A history may be elicited of an infected scratch or wound due to a bone or knife contaminated by animal secretions approximately 5–7 days or, at most, 2 weeks prior to the onset of symptoms (Price and Bennett, 1951). The infected area is swollen. The lesion consists of a well-defined, slightly elevated, violaceous zone that spreads peripherally as discoloration of the central area fades (King, 1946). Systemic effects are uncommon. Low-grade fever and arthralgias occur in approximately 10% of cases and lymphangitis and lymphadenopathy in approximately one third (Nelson, 1955). There may be arthritis of an adjacent joint. Vesicles may be present, but suppuration does not occur. The absence of suppuration along with the violaceous color, lack of pitting edema, and disproportionate pain help to distinguish erysipeloid from staphylococcal or streptococcal infection. Erysipeloid is a self-limited condition, the lesions usually resolving without therapy within 3 or 4 weeks.

DIFFUSE CUTANEOUS FORM This is a rare situation in which the violaceous cutaneous lesion progresses proximally from the site of inoculation or appears at remote areas (Ehrlich, 1946; Grieco and Sheldon, 1970). Bulla formation may occur. The patients often have systemic manifestations such as fever and joint pains, but blood cultures are negative. The clinical course is much more protracted than in the localized disease form, and recurrences are not uncommon. In one instance, a butcher who ate sausage from a pig slaughtered because of swine erysipelas developed widespread urticaria with the rhomboid pattern characteristic of swine erysipelas (Hunter, 1974).

SEPTICEMIA AND ENDOCARDITIS Systemic *E. rhusiopathiae* infection is uncommon. No cases of systemic disease were seen among the 500 cases of erysipeloid described by Nelson (1955) or among the 329 cases reported by Gilchrist (1904). Fifty cases of *E. rhusiopathiae* infection associated with an extremely high incidence of endocarditis (90%) were reported between 1940 and 1988 (Reboli and Farrar, 1989). All reported cases of endocarditis except one involving a Starr-Edwards prosthetic aortic valve (Gransden and Eykyn, 1988) were of native valves. Only about one-third of patients had a history of an antecedent skin lesion or a concurrent characteristic skin lesion of erysipeloid (Gorby and Peacock, 1988). Gorby and Peacock (1988) compared clinical features of *E. rhusiopathiae* endocarditis with those of endocarditis caused by other bacteria (Kaye, 1976). They found a higher male-to-female ratio (which probably reflects occupational exposure), a greater propensity for involvement of the aortic valve, and a much higher mortality rate (38%) among patients with *E. rhusiopathiae* endocarditis. There was more prior heart disease among those with endocarditis caused by other organisms. In nearly 60% of patients, *E. rhusiopathiae* endocarditis apparently developed on previously normal heart valves. The clinical picture with respect to fever, peripheral skin stigmata of endocarditis, emboli, splenomegaly, hematuria, and mycotic aneurysm was similar for the two groups. Very few cases of endocarditis have occurred in immunocompromised patients, but a history of alcohol abuse was present in 33%. The presentation is most often subacute but may be acute (Schiffman and Black, 1956; Simberkoff and Rahal, 1973; Gorby and Peacock, 1988). The most common complication of endocarditis, congestive heart failure, was present in approximately 80% of patients (Russell and Lamb, 1940; Grieco and Sheldon, 1970; McCracken et al., 1973; Heggers et al., 1974; Kramer et al., 1982; Fliegelman et al., 1985). Myocardial abscesses and aortic valve perforation have been reported (Russell and Lamb, 1940; Heilman and Herrell, 1944; Morris et al., 1965; Fowler et al., 1967; Mandal and Malloch, 1971; Kramer et al., 1982). More than one-third of patients required valve replacement (Gorby and Peacock, 1988). Diffuse glomerular nephritis and meningitis have also been reported

as complications (Silberstein, 1965; Simberkoff and Rahal, 1973). Ognibene et al. (1985) reported the first case of septic shock associated with this organism in a patient without convincing evidence of endocarditis. A recent Medline survey on *Erysipelothrix* found very few clinical case reports (Schuster et al., 1993; Hill and Ghassemian, 1997).

OTHER   INFECTIONS Osseous necrosis of the thumb has been reported in a patient who developed fatal endocarditis (Klauder et al., 1943), and Torkildsen (1943) described a case of intracranial abscess. Chronic arthritis has been reported in a few cases in Europe (Ehrlich, 1946).

Treatment

The mainstay of treatment of infections caused by *E. rhusiopathiae* is antibiotic therapy. Although skin lesions usually heal spontaneously within 4 weeks, second attacks may occur and lesions may persist for months. Healing is hastened by antibiotic therapy. Susceptibility data are limited. Most strains are highly susceptible to penicillins, cephalosporins, erythromycin and clindamycin (Heilman and Herrell, 1944; Stiles, 1947; Sneath et al., 1951; Poretz, 1985; Gorby and Peacock, 1988). Minimal inhibitory concentrations for penicillins have been reported to range from 0.0025 to 0.06 µg/ml, with minimal bactericidal concentrations of 0.0025–0.75 µg/ml (Gorby and Peacock, 1988). Susceptibility to chloramphenicol and tetracycline is variable. Most strains are resistant to sulfonamides, trimethoprim-sulfamethoxazole, aminoglycosides, vancomycin, novobiocin and polymyxins. Resistance to vancomycin is noteworthy because this agent is often used in empiric therapy of prosthetic valve endocarditis and in the treatment of native valve endocarditis due to Gram-positive organisms in individuals who are allergic to penicillins.

Penicillin G, in doses of 12–20 million units/day, is the drug of choice for serious infections caused by *E. rhusiopathiae* (Poretz, 1985). Recommended duration of therapy for endocarditis is 4–6 weeks, although shorter courses consisting of 2 weeks of intravenous therapy, followed by 2–4 weeks of oral therapy have been successful (Muirhead and Reid, 1980; Ognibene et al., 1985). There has been no reported experience in the treatment of penicillin-allergic patients. Cephalosporins are the most appropriate alternatives since both clindamycin and erythromycin are only bacteriostatic agents. Valve replacement has been necessary in about one-third of cases of endocarditis.

Pathogenesis and Pathology

The factors responsible for virulence in *E. rhusiopathiae* are not clearly defined. Most strains isolated from pigs with swine erysipelas belong to serotypes 1a, 1b and 2. Serotype 1a is most common in septicemia. Ability to adhere to mammalian cells may be an important determinant of virulence. Virulent strains of *E. rhusiopathiae* adhere much more avidly than avirulent strains to porcine kidney cell lines (Takahashi et al., 1987b), and strains isolated from swine with endocarditis or septicemia adhere more strongly to swine heart valve tissue in vitro than other isolates of *E. rhusiopathiae* (Bratberg, 1981). *Erysipelothrix rhusiopathiae* also produces a hyaluronidase and a neuraminidase, which cleaves α-glycosidic linkages of sialic acid, a mucopolysaccharide on the surface of mammalian cells. Neuraminidase activity is higher in virulent than in avirulent strains (Krasemann and Müller, 1975), and there is evidence that this enzyme may play a role in the pathogenesis of arthritis and thrombocytopenia in rats experimentally infected with *E. rhusiopathiae* (Shinomiya and Nakato, 1985; Nakato et al., 1986).

Intravenous injection of the organism into rabbits is fatal in 2–3 days. An erysipeloid rash develops in the injected ear, the lungs become hemorrhagic, and a pericardial exudate develops. Congestion of the viscera is noted with pinpoint focal necrosis in the liver and mononuclear cell infiltrates in the spleen (Smith, 1983). On histopathological examination, bacilli are scarce. Inoculation of the conjunctiva produces conjunctivitis, which is often followed by fatal disseminated infection. Subcutaneous injections seldom cause death.

The rhomboidal skin lesions described in swine are the result of thrombotic vasculitis of end arterioles (Grieco and Sheldon, 1970). Injection of the organism into swine produces an inflammatory polyarthritis, lymphadenopathy, endocarditis, peripheral monocytosis, and focal necrosis of the liver and myocardium (Smith, 1983). The endocarditis in swine usually involves the mitral valve and there is a tendency for the vegetations to invade the mural endocardium (Russell and Lamb, 1940). Pathological changes in human cases of septicemia and endocarditis are indistinguishable from changes caused by other bacterial organisms.

Fatal septicemia occurred in a wild-caught opossum two months after capture of the animal. *Erysipelothrix rhusiopathiae* was isolated from heart blood, liver, spleen and lungs (Lonigro and LaRegina, 1988). Although no evidence of endocarditis was found, when this strain was inoculated intravenously into previously healthy opossums, all of three animals developed vege-

tations on the atrioventricular valve. Brown and Brenn stains revealed small Gram-positive rods within the vegetations, but the organism could not be grown from blood or vegetations of any of the animals (LaRegina et al., 1988).

Swine arthritis bears some similarities to human rheumatoid arthritis (Sikes, 1958; Grieco and Sheldon, 1970; Phillips, 1986). It is marked by pannus formation with destruction of cartilage at the site of pannus attachment, intra-articular fibrous adhesions, and subchondral cellular reaction (Sikes, 1958; Grieco and Sheldon, 1970). Antigens of *E. rhusiopathiae* have been detected by electron microscopy and immunofluorescence in deep joint tissues of swine as long as 18 months after infection. It is not known whether development of chronic arthritis is due to specific immune reactions against *E. rhusiopathiae* or to autoimmune reactions or both (Wood, 1984).

Bacteriology

Morphology, Growth, and Biological and Chemical Properties

The cell wall of *E. rhusipathiae* contains a peptidoglycan based on lysine (Feist, 1972). More detailed analysis of the peptidoglycan structure using the method described by Schleifer and Kandler (1972) and Schubert and Fiedler (2001) indicate that the hallmark of *Erysipelothrix* is the presence of a B-cell wall type in which the peptide bridge is formed between amino acids at positions 2 and 4 of adjacent peptide side chains and not, as in the vast majority of bacteria, between amino acids at positions 3 and 4. The B-type occurs not only in *Erysipelothrix* but also in some other members of the Clostridium subphylum, e.g., *Holdemania*, *Acetobacterium*, *Clostridium barkeri* and *Eubacterium limosum*, and

within the family Microbacteriaceae, order Actinomycetales (Schleifer and Kandler, 1972). The two-dimensional thin-layer chromatogram of the partial acid hydrolysate of strain MF-EP02[T] revealed (besides the presence of lysine, glutamic acid, glycine, serine, alanine, muramic acid and glucosamine) the fragments D-Glu→Gly, L-Ser→D-Glu and L-Lys→L-Lys, but no aspartic acid or fragments containing aspartic acid. The quantitative amino acid composition (Mackenzie, 1987) is Ala:Gly:Ser:Glu:Lys = 1.7:0.7:0.9:1.0:1.5. It can thus be deduced that the peptidoglycan type is B1δ with the interpeptide bridge being Gly→L-Lys→L-Lys. The peptidoglycan composition (identical in the type strains of all three *Erysipelothrix* species) differs from that of *Holdemania filiformis* [{ATCC 51649}][T] [{genbank}{Y11466}] (the nearest, although remote, neighbor), which has L-Asp→L-Lys interpeptide bridges (Willems et al., 1997).

Several sugars occur in the cell wall (arabinose, galactose, glucose, glucose-6-phosphate, galactose-6-phosphate, ribose and xylose). Monosaccharide patterns show that there are three chemovars, which do not correlate with the serovars (Feist, 1972).

Mycolic acids are not present. Cellular fatty acid composition (mainly used to identify asporogenous, aerobic Gram-positive rods) of *E. tonsillarum* and/or *E. rhusiopathiae* has been reported by Julak et al. (1989), Bernard et al. (1991), von Graevenitz et al. (1991), and Takahashi et al. (1994). The most comprehensive fatty acid methyl ester (FAME) analysis of *Erysipelothrix* (Verbarg et al., 2004) was in extracts of methanolysates and was performed using the MIDI microbial identification system as described by Sasser (1990). The composition of fatty acids (Table 1) reveals high similarity of all strains of the *Erysipelothrix* species. A dendro-

Table 1. Fatty acid composition (>1% of total) of Erysipelothrix spp.

Fatty acid	E. rhusiopathiae DSM 5055[T] and three other strains[a] (%)	E. tonsillarum DSM 14972[T] (%)	E. inopinata DSM 15511[T] (%)
10:0	—	—	2.96
14:0	1.59–1.90	2.66	2.95
cis-8-16:1	1.0–1.25	—	1.24
16:0	24.2–31.7	28.2	34.2
17:0	1.0–1.30	1.35	1.21
18:2	6.46–7.37	5.04	2.77
cis-9-18:1	30.15–39.33	32.51	33.12
cis-11-18:1	1.03–1.94	—	—
cis-12-18:1	4.83–5.85	5.84	2.53
18:0	10.18–19.95	19.64	12.88
20:4	1.11–1.44	1.47	—

Symbol and abbreviations: —, ≤1% of total fatty acids; [T], type strain; and DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen.

[a]*Erysipelothrix rhusiopathiae* DSM 5056, DSM 5057, and DSM 5058.

Fig. 2. Dendrogram based on Euclidian distances determined from comparison of fatty acid patterns.

Erysipelothrix inopinata DSM 15511[T]

Erysipelothrix tonsillarum DSM 14972[T]

Erysipelothrix rhusiopathiae DSM 5057

Erysipelothrix rhusiopathiae DSM 5056

Erysipelothrix rhusiopathiae DSM 5055[T]

Erysipelothrix rhusiopathiae DSM 5058

Euclidian distance
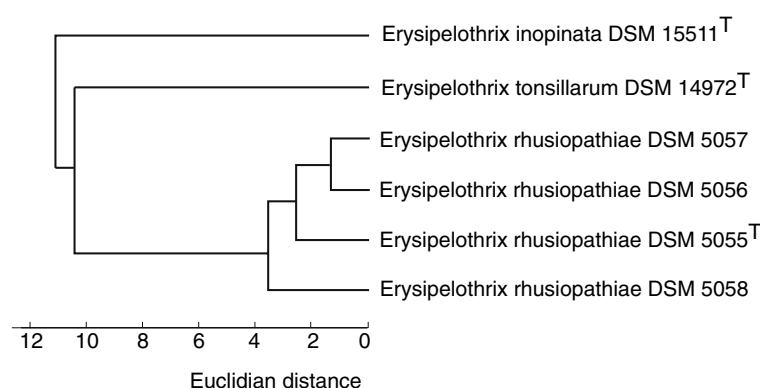
gram of FAME relationships is depicted in Fig. 2. The pattern is dominated by C18:1-*cis*-9 (>30%), C16:0 (>24%) and C18:0 (>10%) FAMEs. This pattern differs from that of *Holdemania filiformis* which contains higher amounts of C18:1-*cis*-9 (50%), additional minor components and significant amounts of dimethyl acetal (C18:1-*cis*-9 [12%] and C16:0 [4%]) (Willems et al., 1997). Figure 2 is a dendrogram of relationship based on Euclidian distance estimated from a comparison of fatty acid methyl ester patterns.

L-Forms of *E. rhusiopathiae* have been described (Stuart, 1972a; Pachas and Currid, 1974; Todorov, 1976).

None of the *Erysipelothrix* strains contained significant amounts of menaquinones, no matter which growth conditions were used for the preparation of isoprenoid quinones (aerobic and anaerobic growth conditions on trypticase soy broth [TSB] agar + 5% sheep blood). Only traces of menaquinone (MK)-7 were detected in cells of *E. tonsillarum* DSM 14972[T] following aerobic cultivation (Collins and Jones, 1981; Verbarg et al., 2004). Isoprenoid quinones extraction by chloroform/methanol (2 : 1, v/v) from lyophilized cells, purification by preparative TLC on silica gel, and analyses by HPLC followed described methods (Collins et al., 1977; Groth et al., 1996).

Metabolic properties of type strains of *Erysipelothrix* species, determined by using the API 32 STREPT and BIOLOG GP microplate panel are indicated in Table 2.

CHARACTERIZATION OF *ERYSIPELOTHRIX RHUSIOPATHIAE* On blood agar this organism may be α-hemolytic but never β-hemolytic (see Table 3). There is a dual colonial and microscopic appearance (Figs. 3 and 4). After growing 24–48 h at 37°C on trypticase soy agar containing 5% sheep blood, colonies (known as smooth or S-forms) are very small (0.1 mm in diameter), convex, circular and transparent with a smooth glistening surface and edge. In older cultures, colonies are slightly larger and have opaque centers. Larger (0.2–0.4 mm diameter) flatter colonies with a matte surface and fimbriated edge are R-form or rough colonies. They may resemble miniature *Bacillus anthracis* colonies (Wilson and Miles, 1975). Both forms are usually light blue or sometimes green when viewed with oblique illumination. Intermediate forms are also seen (Barber, 1939; Ewald, 1981). S-form colonies dissociate to give rise to intermediate and R-form colonies. R-form colonies also give rise to S-forms (Ewald, 1962; Wawrzkiewicz, 1964). During these changes in morphology and cultural characteristics, there are also changes in virulence and antigenic properties. (The type-specific antigens remain unchanged.) In broth, S-form organisms cause a slight turbidity and a powdery deposit; R-forms have a tangled hair-like appearance. Microscopically, S-form organisms are the small, straight or slightly curved rods characteristic of the genus and measure 0.3–0.6 μm by 0.8–2.5 μm, while R-form organisms form long nonbranching filaments which may have a beaded appearance and which can be greater than 16 μm in length. Both forms stain evenly and may show deeply stained granules. Morphology varies with the medium, pH, and temperature of incubation. Acidic pH and temperature of 37°C favor R-forms (Wilson and Miles, 1975). Alkaline pH (7.6–8.2) and temperature of 30°C favor S-forms (Grieco and Sheldon, 1970). S-form organisms are seen in smears from blood and tissue and in acute forms of illness such as sepsis; R-forms (or S-forms and R-forms) are seen in more chronic conditions such as endocarditis or arthritis (Ewald, 1981).

Growth of *E. rhusiopathiae* is improved by blood or 5–10% serum, tryptophan and glucose. The best growth occurs in 0.1% glucose broth or on 0.5% glucose agar. Larger amounts of glucose may be inhibitory. Glucose metabolism is via the Embden-Meyerhof-Parnas pathway, with a small amount by the hexose monophosphate shunt (Robertson and McCullough, 1968). Exogenous

Table 2. Phenotypic properties of type strains of *Erysipelothrix* species.[a]

	E. inopinata	*E. rhusiopathiae*	*E. tonsillarum*
API STREPT[b]			
β-Glucosidase	+	−	+
Alkaline phosphatase	−	−	+
Ribose (acid)	w	−	+
Lactose (acid)	−	+	−
Trehalose (acid)	+	−	−
N-Acetyl-β-glucosamidase	+	+, some −	+
Biolog GP[c]			
Utilization of			
β-Mannosidase	w	−	−
L-Arabinose	−	+	w
N-Acetyl-D-mannosamine	−	+	+
Arbutin	+	−	−
Cellobiose	+	−	−
D-Fructose	−	+	+
D-Galactose	−	+	+
Gentiobiose	+	−	−
α-D-Lactose	−	+	−
D-Mannose	−	+	−
3-Methyl glucose	−	−, some +	ND
D-Psicose	−	+	ND
D-Ribose	w	+, type −	ND
Salicin	+	−	ND
D-Trehalose	+	−	ND
Xylose	−	+, type −	ND
Glycerol	+	−	ND

Symbols and abbreviations: +, positive; −, negative; w, weak; ND, not determined.

[a]As determined by API 32 STREPT and BIOLOG GP microplate panel.

[b]According to API 32 STREPT, all strains were positive for glycyl-tryptophan arylamidase, pyroglutamic acid arylamidase, and for acid production from glucose. All strains were negative for oxidase, aminopeptidase, hydrolysis of starch, gelatin liquefaction, DNA and casein, urease, acid from mannitol, sorbitol, raffinose, sucrose, L-arabinose, D-arabitol, cyclodextrin, glycogen, pullulan, maltose, melibiose, melezitose, tagatose, β-glucoronidase, production of acetoin and hydrolysis of hippurate.

[c]As determined with Biolog GP, all strains use the following substrates: adenosine, uridine, methylpyruvate, *N*-acetylglucosamine and α-D-glucose. All strains are negative in β-methyl-D-glucoside, D-tagatose, lactamide, alaninamide, D-arabitol, lactulose, α-methyl-D-mannoside, D-lactic acid methyl ester, D-alanine, β cyclo-dextrin, maltose, palatinose, turanose, L-lactic acid, L-alanine, dextrin, maltotriose, xylitol, D-malic acid, L-asparagine, glycogen, D-mannitol, D-raffinose, L-malic acid, inulin, L-fucose, L-rhamnose, acetic acid, L-glutamic acid, adenosine-5′-monophosphate, mannan, D-melezitose, α-hydroxy-butyric acid, mono-methyl succinate, glycyl-L-glutamic acid, thymidine-5′-monophosphate, Tween 40, D-galacturonic acid, D-melibiose, β-hydroxy-butyric acid, propionic acid, L-pyroglutamic acid, uridine-5′-monophosphate, Tween 60, α-methyl D-galactoside, sedoheptulosan, γ-hydroxy-butyric acid, pyruvic acid, L-serine, fructose-6-phosphate, D-gluconic acid, β-methyl D-galactoside, D-sorbitol, *p*-hydroxy-phenyl acetic acid, succinamic acid, putrescine, glucose-1-phosphate, stachyose, α-keto glutaric acid, succinic acid, 2,3-butanediol, glucose-6-phosphate, amygdalin, *m*-inositol, α-methyl D-glucoside, sucrose, α-keto valeric acid, *N*-acetyl L-glutamic acid and D-L-α-glycerol phosphate.

citrate is not used. Fermentative activity is weak (Smith, 1983). The fermentation pattern varies with the basal medium used (White and Shuman, 1961; Wood, 1970). Acid production from carbohydrates is usually poor or inconsistent when in 1% peptone water. It is recommended that 5–10% horse serum be added to the basal medium (White and Shuman, 1961; Wood, 1970; Seeliger, 1974), but since this is not always convenient, one may test for acid production in nutrient broth with 0.5–1% of the test carbohydrate and phenol red added as an indicator. In addition to lactic acid, small amounts of acetic acid, formic acid, ethyl alcohol, and CO_2 are produced from

the fermentation of glucose. Acid without gas is produced within 48 h from glucose, lactose, fructose and galactose (Karlson and Merchant, 1941). A few strains produce acid from maltose fermentation in 6–7 days. Xylose, mannitol, and sucrose are not fermented. This genus is indole and Voges-Proskauer negative and usually methyl red negative (Sneath et al., 1951). The methyl red test may be weakly positive when performed in a broth containing peptone, yeast extract, glucose, potassium phosphate, magnesium sulfate, and manganese sulfate (Wilkinson and Jones, 1977). There is no discoloration of methylene blue milk, little or no change in litmus

Table 3. Additional characteristics of *Erysipelothrix rhusio-pathiae* and *E. tonsillarum*.

Test	*E. rhusiopathiae*[a]
Aerobic growth	+*
Anaerobic growth	+*
α-Hemolysis	+[ND]
β-Hemolysis	−[ND]
Acid from glucose, fructose, galactose, lactose	+*, +[ND], +*, +*
Acid from maltose, xylose, mannitol, and sucrose	−*, −[ND], −[ND], −[w]
Gas from glucose, fructose, galactose, and lactose	−[ND]
H$_2$S in TSI	+*
Nitrate reduction	−[ND]
Indole	−[ND]
Voges-Proskauer	−[ND]
Methyl red	−[ND]
Methylene blue milk	−[ND]
Litmus milk	v[ND]
Gelatin stab	Test-tube brush or "pipe cleaner" growth*
Growth at 4°C	−[ND]
Susceptibility to NaCl (8.5%)	+[ND]
Susceptibility to neomycin	−[ND]

Symbols and abbreviations: +, positive; −, negative; w, weak; v, variable; and TSI, triple sugar iron medium.
[a]Reactions of *E. tonsillarum* (Takahashi et al. 1987a) identical to those of *E. rhusiopathiae* are indicated as superscripts: * and [w], weak reaction. [ND], not determined.
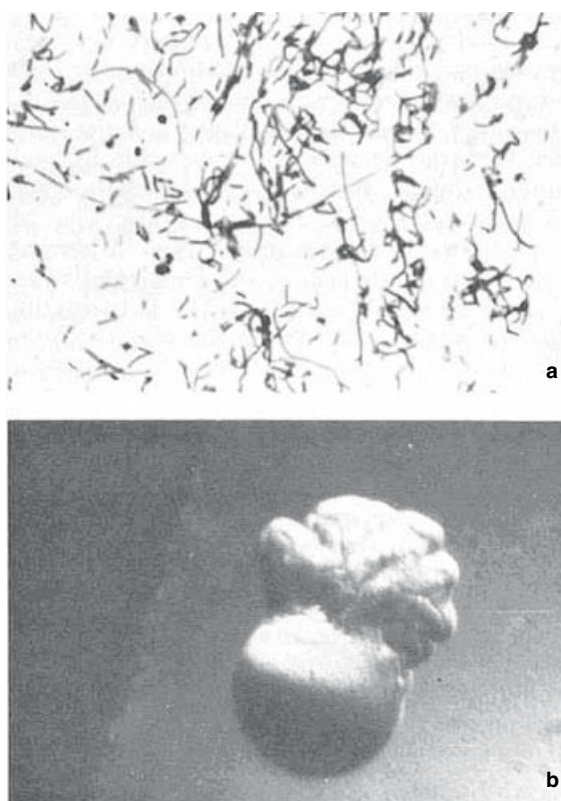
Fig. 4. *Erysipelothrix rhusiopathiae*, smooth and rough forms.

Fig. 3. *Erysipelothrix rhusiopathiae*, smooth form.

milk, and no reduction of nitrate. Urea, esculin, sodium hippurate, starch, cellulose and casein are not hydrolyzed. Xanthine and tyrosine are not degraded. The majority of strains produce hydrogen sulfide, but results can vary with the medium used (Ewald, 1964). Hydrogen sulfide production is an important test. It is best carried out on triple sugar iron agar (TSI) slants, in which hydrogen sulfide causes a blackened butt (Wood, 1970). An occasional old laboratory strain does not produce detectable hydrogen sulfide in TSI agar. Gelatin stab cultures yield a very characteristic pattern of growth described as a "test tube brush" or a "pipe cleaner" (Ewald, 1964; Weaver, 1985; Jones, 1986). After 24 h, growth is faint, hazy and limited to an area just below the surface. Within a few days, however, growth extends in a column to the bottom of the tube. There is no liquefaction of the gelatin. S-forms produce fine horizontal outgrowths that extend only 2–3 mm from the stab in a typical "pipe-cleaner" pattern (Ewald, 1964). R-forms extend further out and look like a "test-tube brush" (Fig. 5). This test is not convenient for most laboratories to do since the gelatin must be incubated at 22–25°C to maintain its solid state. Furthermore, this test is not required for identification.
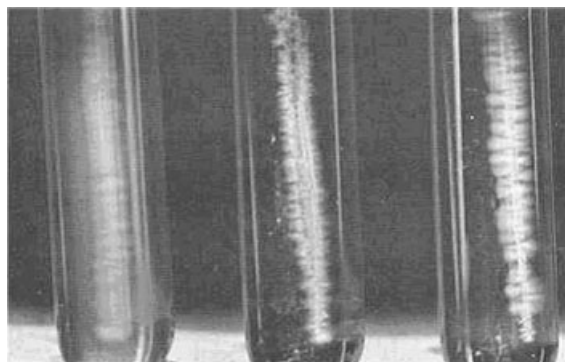
Fig. 5. Three *Erysipelothrix* strains on ferrochloride-gelatin medium after incubation of 48 h at 20°C. On the left, smooth strain; in the middle and on the right, dissociated strains.

Most known strains produce hyaluronidase (Ewald, 1957). There may be a correlation between production of this enzyme and virulence. Good producers of hyaluronidase usually belong to serovar 1 (Ewald, 1957; Ewald, 1981). All strains produce neuraminidase but in varying amounts. There is a good correlation with virulence (Krasemann and Müller, 1975; Müller and Seidler, 1975; Müller and Krasemann, 1976; Nikolov and Abrashev, 1976). There are also virulent strains that do not produce neuraminidase or which produce small amounts. Synthesis of this enzyme depends on the growth phase of the bacteria. There is maximum production at the end of the logarithmic phase of growth (Abrashev and Zamfirova, 1976).

Characterization of *Erysipelothrix Tonsillarum* (Takahashi et al., 1987a) In addition to the genus specific properties and characters listed in Table 2, the rods are approximately 0.3 μm wide by 1.0–1.5 μm long. Surface colonies on brain heart infusion (BHI) agar after 2 days of incubation are punctiform to approximately 1 mm in diameter, circular, entire, convex, colorless, transparent and soft. Though *E. tonsillarum* is described to be morphologically and biochemically indistinguishable from the 10 strains of *E. rhusiopathiae* included in the study by Takahashi et al. (1987a), the two species differ in their acid production from maltose (*E. rhusiopathiae* is negative, *E. tonsillarum* positive). In addition to reactions indicated in Table 2, acid production is obtained from dextrin and levulose; weak acid production from mannose; and no acid from glycerol, salicin, inositol, dulcitol, raffinose, mannitol, rhamnose, trehalose, xylose, sorbitol and arabinose. Litmus milk is not acidified. *Erysipelothrix tonsillarum* isolates are susceptible to penicillin G, ampicillin, and erythromycin but not to kanamycin and sulfadi-methoxine.

Characterization of *Erysipelothrix Inopinata* (Verbarg et al., 2004) In addition to the genus specific properties and characters listed in Table 2, the rods are approximately 0.5 μm wide and 1.5–3.0 μm long. Surface colonies on BHI (Difco) after 2 days of incubation are punctiform to approximately 1.5 mm in diameter, creamy white, undulate, convex, translucent and soft. Growth occurs under aerobic and anaerobic conditions in BHI and Columbia blood media, preferably at pH 8. Growth occurs at 20°C and 40°C, but not at 45°C. The optimal temperature for growth is 25–30°C. The mol% G+C of DNA is 37.5 (HPLC). Type strain is *Erysipelothrix inopinata* DSM 15511^T (CIP 107935^T).

Specimen Collection, Transport, and Maintenance

Routine blood culture techniques are adequate for specimen collection and organism growth in suspected cases of sepsis or endocarditis (Muirhead and Reid, 1980). Because organisms are located only in deeper parts of the skin in cases of erysipeloid, aspirates or biopsy specimens from the edge of the lesion are needed to obtain the organism (Price and Bennett, 1951). Biopsy should be of the entire thickness of the dermis. The organism may also be obtained from cases of erysipeloid by injecting saline into the edge of the lesion and aspirating some of the injected saline for culture (Smith, 1983).

Immediately after collection, the specimen should be put into an infusion broth of 1% glucose and kept at room temperature or refrigerated until it reaches the laboratory. Cultures can be maintained for several months by stab inoculation into tubes of nutrient agar (pH 7.4; Jones, 1986). Freeze drying or freezing on glass beads at −70°C (Feltham et al., 1978) as well as storage under liquid nitrogen, is appropriate for long-term maintenance.

In many countries, *E. rhusiopathiae* is classified as a risk group 2 pathogen and specific rules and regulations apply for its shipment at the national and international level (see Rohde, 1999).

Isolation and Identification

Commercially available blood culture media are satisfactory for primary isolation from blood since *E. rhusiopathiae* is not particularly fastidious. Biopsy specimens or tissue aspirates from skin lesions should be put into infusion broth with 1% glucose and incubated in air or in 5–10% carbon dioxide at 35–37°C (Weaver, 1985). At 24-h intervals, subcultures to blood agar plates should be made. No visible growth occurs

on potato or MacConkey's medium. Use of selective media is not necessary unless the specimen is heavily contaminated with other bacteria such as those from soil, manure, or animal tissues (Wood, 1965; Wood and Packer, 1972). Many selective media have been described, including medium ESB (*Erysipelothrix* selective broth), a nutrient broth containing horse serum, kanamycin, neomycin and vancomycin (Wood, 1965), and a tryptose blood agar containing crystal violet and sodium azide (Packer, 1943). Another selective liquid enrichment for *E. rhusiopathiae* contains kanamycin, crystal violet, sodium azide, and liquified phenol (Bohm, 1971). A solid medium that is a modification of this enrichment contains water blue (aniline blue) and sucrose (Bohm, 1971). Since *E. rhusiopathiae* does not ferment sucrose, colonies appear colorless on the water blue sucrose agar. In cases of chronic infection in which the number of bacteria is small, enrichment by the addition of horse, calf or swine serum in broth and incubation for longer than 10 days may be necessary.

Selective Media for Erysipelothrix Modified ESB Medium (Wood, 1965)

Nutrient broth no. 2 (Oxoid) dehydrated	25 g
Distilled water	1 liter
Horse serum	50 ml
Kanamycin	400 mg
Neomycin	50 mg
Vancomycin	25 mg

Place specimen in 10 ml of modified ESB medium and incubate overnight at 35°C. Remove 5 ml, centrifuge at 1400×g for 20 min, resuspend the sediment in 1-2 ml of 0.8% saline, and plate a sample on MBA medium. After incubation at 35°C for 24–48 h, examine the plate for colonies.

MBA Medium (Harrington and Hulse, 1971)

Heart infusion agar (Difco)	40 g
Sodium azide	0.4 g
Distilled water	1 liter
Horse blood or	20 ml
Horse serum	50 ml

Packer's agar (Packer, 1943) is recommended for grossly contaminated specimens such as feces or soil because it is more selective for *E. rhusiopathiae* than is MBA medium (Jones, 1986).

Phenotypic identification of the organism is based on the results of Gram stain, lack of motility, hydrogen sulfide production, indole production, catalase activity, growth on agar containing potassium tellurite, and hemolysis on blood agar. The result of any one test is insufficient for identification. *Erysipelothrix rhusiopathiae* will need to be differentiated from other Gram-positive bacilli, in particular from *Actinomyces* (*Corynebacterium*) *pyogenes* and *Arcanobacterium*

(*Corynebacterium*) *haemolyticum*, and from *Listeria monocytogenes*. These other organisms are β-hemolytic on blood agar and do not produce hydrogen sulfide in the butt of TSI slants. *Listeria monocytogenes* is catalase positive and motile. The neomycin susceptibility test can be used to distinguish *E. rhusiopathiae* from *Listeria monocytogenes*, the former being resistant to neomycin and the latter being susceptible (Fuzi, 1963). Some *Bacillus* species and streptococci form hydrogen sulfide, but they can be differentiated from *E. rhusiopathiae* by formation of spores and by cellular morphology, respectively. *Erysipelothrix rhusiopathiae* has occasionally been misidentified as a viridans streptococcus (Procter, 1965; Gorby and Peacock, 1988). It has also been dismissed as a contaminant.

The mouse protection test is considered the best method for confirming an isolate as *E. rhusiopathiae* (Jones, 1986). In this test, a subcutaneous injection of 0.1 ml from an 18- to 24-h broth culture of the suspected *E. rhusiopathiae* is administered to mice along with a dose of commercial equine hyperimmune *E. rhusiopathiae* antiserum at another site. A control group of mice is injected with the broth culture but not the antiserum. If the organism is *E. rhusiopathiae*, the mice that did not receive antiserum die in 5–6 days, but those receiving antiserum are protected (Weaver, 1985). This test detects only those strains that are virulent for mice, but since most strains are virulent it is a good confirmatory test. One may also inject suspect clinical material subcutaneously into mice and isolate the organism from the kidneys or spleen when they die a few days later (Ewald, 1981).

Erysipelothrix rhusiopathiae has heat- and acid-stable, type-specific antigens and heat-labile, species-specific antigens (Kalf and Grece, 1964; Grieco and Sheldon, 1970; Jones, 1986). The type-specific antigens are polysaccharides. Strains may be identified serologically. In 1949, Dedié proposed that the two main serovars be designated as "A" and "B" and that strains which showed no reaction with A- or B-type-specific antiserum be designated as "N." New serovars in group N were designated by consecutive letters of the alphabet. One of the problems with this system was that different serological methods were used. Kucsera (1973) recommended the use of a double agar-gel diffusion precipitin test using autoclaved antigens and type-specific antisera and a uniform system for designating serovars using Arabic numbers. This numerical system is preferred over the older alphabetical system. Serovars 1 and 2 correspond to A and B of Dedié's system. These are the most common of the 22 serovars (Wood et al., 1978; Norrung, 1979). Strains of human and animal origin are

antigenically alike (Sneath et al., 1951). Most virulent strains causing acute infection belong to serovar 1 (or A). Strains of serovar 2 (or B) have been isolated primarily from chronic cases.

Direct and indirect fluorescent-antibody tests are also available in lieu of the mouse protection test to confirm identification of *E. rhusiopathiae* (Avilag et al., 1972; Heggers et al., 1974). They can be used to detect *E. rhusiopathiae* in tissues (Dacres and Groth, 1959; Seidler et al., 1971) and in enrichment broth (Harrington et al., 1974). In general, serological tests are not practical for routine use in a clinical laboratory for identification of the organism or for detection of antibody in patient sera. Bacteriophages active on *E. rhusiopathiae* strains have been isolated (Valerianov et al., 1976).

Identification schemes for *Erysipelothrix* strains, especially in comparison with other rapidly and aerobically growing Gram-positive rods, coryneforms and streptococci have been published by von Graevenitz and Funke (1996), Soto et al. (1994), and Freney et al. (1992). Potential errors in the recognition of *E. rhusiopathiae* have been summarized by Dunbar and Clarridge (2000).

The original seven *E. tonsillarum* strains studied (Takahashi et al., 1987a; 1987b) originated from pigs raised on different farms in different locations in Japan. Strains were cultivated on BI broth culture and BI agar (pH 7.6). Phenotypically strains of this species resemble those of *E. rhusiopathiae*. However, the virulence (measured by LD_{50}; the dose that kills 50% of the population) for mice is usually higher for *E. rhusiopathiae* strains ($1.0–25 \times 10^0$) than for *E. tonsillarum* strains (1.0×10^0 to 6.9×10^5). All but one *E. tonsillarum* strain in the study of Takahashi et al. (1987a) were serotype 7, showed no erythema at skin injection sites, and were nonsystemically pathogenic.

Erysipelothrix inopinata was isolated as a contaminant in a vegetable based growth medium. In the course of validating production processes involved in industrial aseptic manufacture of pharmaceuticals, a vegetable based growth medium was tested for its effect on dilution performance. Dehydrated CSB medium (peptone vegetable, 20.0 g; D(+)-glucose, 2.5 g; K_2HPO_4, 2.5 g) was added to 1 litre distilled water and the solution filtered through a membrane filter (pore width, 0.2 μm). A medium sample incubated at room temperature for 3 days became turbid. Microscopic analysis and plating in medium TSA (tryptic soy agar: casein peptone, 15 g; soy peptone, 5 g; NaCl, 5.0 g; agar 15.0 g; and water, 1 liter, pH.7.3) and TSS (TSA + 5% sheep blood) indicated the presence of a single contaminant, DSM 15511[T].

MOLECULAR IDENTIFICATION Analysis of the 16S rRNA gene sequence will allow unambiguous affiliation of an isolate to the genus. The high sequence similarity found for *E. rhusiopathiae* and *E. tonsillarum*, however, will not allow the affiliation to either of these species. Identification of members of the genus *Erysipelothrix* has been proposed on the basis of a polymerase chain reaction (PCR) system, using a selective primer pair that amplifies a 407-bp DNA segment of the 16S rRNA gene (Makino et al., 1994). In silico analysis of the 16S rRNA gene sequence of *E. inopinata* indicates that this species too will be identified (Verbarg et al., 2004). Another PCR system has been described for the amplification of the rDNA gene cluster of *Erysipelothrix rhusiopathiae* and *E. tonsillarum* (Takeshi et al., 1999). PCR amplification was able not only to distinguish between the two species but also to discriminate between strains of serovar 2, 13 and 18.

Affiliation of strains to described species by DNA-DNA hybridization studies has been published by Takahashi et al. (1992).

Analysis of nucleic acid patterns has been performed by low molecular mass RNA fingerprinting (Collins-Thompson et al., 1991), pulsed-field gel electrophoresis (Ahrne et al., 1995; Okatani et al., 2001), and automated ribotyping, carried out with the RiboPrinter microbial characterization system (Qualicon; DuPont). The RiboPrint pattern of confirmed the 16S rRNA gene similarities between *E. rhusiopathiae* and *E. tonsillarum* and the more separate position of *E. inopinata* (Verbarg et al., 2004).

At the protein level, various methods have been applied in the discrimination of *Erysipelothrix* strains, including computerized comparison of proteins (Bernath et al., 1997; Bernath et al., 2001), sodium dodecylsulfate (SDS) gel electrophoresis of whole cell proteins (Tamura et al., 1993), multilocus enzyme electrophoresis (Chooromoney et al., 1994), and enzymatic profiling (Takahashi et al., 1989).

ANALYSES OF GENES OTHER THAN RRNA GENES Table 4 compiles sequenced non-*rrn* genes and oligonucleotides, targeting medically relevant genes, some of which are patented but their accession numbers and sequences indicated in public databases (e.g., EBI EBI is the European Bioinformatics Institute, Cambridge, UK (www.ebi.uk.uk)). Other genes, such as housekeeping genes, can be used to construct phylogenetic trees or included in multi-locus-sequence analyses.

Table 4. Examples of genes other than *rrn* genes sequenced from *Erysipelothrix* spp.

Gene sequenced	Comment	Patent or accession number	References
E. rhusiopathiae protective antigen	Collecting and purification	JP2002034568[a]	
E. rhusiopathiae mutant YS-1	Creation of attenuated strains and differentiation of strains	JP1999151084-A/1[a]	
E. rhusiopathiae capsule forming genes	Creation of attenuated strains and differentiation of strains	JP1999000171-A/1[a]	
E. rhusiopathiae unassigned DNA	Recombinant subunit vaccine	JP2000227 9179-A/3[a]	
E. rhusiopathiae SpaA	Protective antigen	AB012763[b]	Makino et al., 1998[c]
E. rhusiopathiae hemolytic protein	Transposase-like protein	AB017185[b]	Makino et al., 1999
E. rhusiopathiae nanH	Sialidase	AB019122[b]	Imada, 1998[c]
E. rhusiopathiae ssaB homologue	Saliva-binding protein	AB019123[b]	Imada, 1998[c]
E. rhusiopathiae Tet (M)	Tetracycline resistance	AB039845[b]	Yamamoto et al., 2000[c]
E. rhusiopathiae rspA, rspB	Adhesive surface proteins	AB052682[b] AB037177[b]	Shimoji et al., 2003
E. rhusiopathiae pAP1	Plasmid	AF028735[b]	Pomerantsev et al. 1997[c]
E. rhusiopathiae rnpB	RNAase P	AF295989[b]	Harris et al., 2001
E. tonsillarum Cpn60	Chaperonin	AY123730[b]	Goh et al. 2002[c]
E. rhusiopathiae DnaK	HSP70	M98865[d]	Partridge et al., 1993
E. rhusiopathiae ew1A	Lipoprotein	U52850[d]	Anitori et al., 1996[c]

Abbreviations: SpaA, truncated surface protective antigen; and HSP70, heat shock protein.

[a]Japanese patent number.

[b]Genbank accession number.

[c]Unpublished.

[d]European Molecular Biology Laboratory (EMBL) accession number.

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CHAPTER 1.2.14

The Genus *Gemella*

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Introduction

The genus *Gemella* until recently consisted of two species, *G. haemolysans* (the type species of the genus), and *G. morbillorum* (Berger, 1992). In the past few years four other species, *G. bergeri* (Collins et al., 1998b), *G. sanguinis* (Collins et al., 1998a), *G. palaticanis* (Collins et al., 1999) and *G. cuniculi* (Hoyles et al., 2000), have been assigned to the genus. The cells of gemellae are cocci, which are arranged in pairs, tetrads, small irregular clusters and sometimes in small chains; elongate and rod-shaped forms may also occur. Cells stain Gram positive but often decolorize easily and appear Gram negative. The organisms are facultatively anaerobic, preferring an atmosphere rich in CO_2, but growth can occur under both aerobic and anaerobic conditions (Berger, 1992). Gemellae are nonmotile and do not form endospores. They are cytochrome oxidase- and catalase-negative.

The genus *Gemella* was originally monospecific, containing only *G. haemolysans*. Because the organism was easily decolorized, it was originally classified as a species of the genus *Neisseria* by Thjøtta and Bøe (1938). These workers recovered the organism from the sputum of a patient with chronic bronchitis, and it was named *N. haemolysins* because of its hemolytic behavior on rabbit blood. The species however phenotypically differed markedly from other members of the *Neisseria* genus, and Berger therefore created the genus *Gemella* (meaning "a little twin") to accommodate the species (Berger, 1960b; Berger, 1961).

Originally *Gemella* was proposed as an aerobic, oxidase-negative, catalase-negative genus within the family Neisseriaceae, but its high sensitivity to penicillins and its resistance to aminoglycosides and polymyxins were more consistent with an affinity to the Gram-positive streptococci (Berger, 1960a). Later studies by Reyn et al. unequivocally showed *G. haemolysans* was incompatible with the family Neisseriaceae (Reyn et al., 1966; Reyn et al., 1970). Electron microscopy studies showed *G. haemolysans* possessed a Gram-positive type cell wall and its DNA G+C content was found to be lower than those of true *Neisseria* species and other genera within the *Neisseriaceae*. Consequently *Gemella* was included in the family Streptococcaceae in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (Reyn, 1974). Later Stackebrandt et al. (1982), on the basis of 16S rRNA cataloguing studies, found *G. haemolysans* neither to be related to Neisseriaceae nor to Streptococcaceae but formed an individual line of descent among the low DNA G+C content Gram-positive bacteria.

A second species, *G. morbillorum*, was added to the genus *Gemella* by Kilpper-Bälz and Schleifer (1988). This organism was first isolated using anaerobic culture methods from patients with measles (Tunnicliff, 1917) and named "*Diplococcus rubeolae*" (Tunnicliff, 1933). This name was subsequently withdrawn (Tunnicliff, 1936) and Prévot's epithet for the same organism, *Diplococcus morbillorum*, was adopted. The species was transferred to the genus *Peptostreptococcus*, as *Peptostreptococcus morbillorum* by Smith (1957), and later Holdeman and Moore (1974) considered the species to be an anaerobic to aerotolerant streptococcus, and reclassified the species as *Streptococcus morbillorum*. Facklam and colleagues observed the close morphological and physiological similarity between *S. morbillorum* and *G. haemolysans* and considered these organisms to be synonyms (Facklam and Smith, 1976; Facklam, 1984). Berger and Pervanidis (1986) also found *S. morbillorum* and *G. haemolysans* were physiologically closely related, but since the cells of both species divide in two planes at right angles, they considered this precluded their assignment to the *Streptococcus* genus. *Streptococcus morbillorum* was finally transferred to the genus *Gemella* on the basis of physiological and phylogenetic evidence (Kilpper-Bälz and Schleifer, 1988), a placement confirmed by comparative 16S rRNA sequencing (Whitney and O'Connor, 1993). In the past few years, four other new species have been assigned to the *Gemella* genus viz, *G. bergeri* and *G. sanguinis* from human clinical sources (Collins et al., 1998a; Collins et al., 1998b) and *G. palaticanis* and *G. cuniculi* from animals (Collins et al., 1999; Hoyles et al., 2000). It is now known that all six

Gemella species form a robust rRNA cluster within the low G+C *Clostridium* subphylum, and do not display a particularly close phylogenetic affinity with any described Gram-positive, catalase-negative genus (Collins et al., 1999; Hoyles et al., 2000). A tree showing the phylogenetic relationships of gemellae and their nearest relatives is shown in Fig. 1.

Habitats

Gemella haemolysans and *G. morbillorum* are commensals of the mucous membranes of humans and some other warm-blooded animals. Although the habitat of the other two *Gemella* spp. from humans, *G. bergeri* and *G. sanguinis*, are not known, it seems likely that they occupy similar niches. *Gemella haemolysans* has been isolated from the oral cavity and the upper respiratory tract of healthy people. The organism has been recovered from the nasopharyngeal mucosa of 25–30% of people sampled (Berger and Wezel, 1960c; Berger, 1985). It has also been recovered from human dental plaque (de Jong and van Hoeven, 1987) and the intestines of pigs (Molitoris et al., 1986). *Gemella morbillorum* is also found in the human oral cavity and upper respiratory tract (Kannangara et al., 1981; Kolenbrander and Williams, 1983; Dzink et al., 1984). In addition, *G. morbillorum* is also found in the human intestinal tract where it is reported to represent approximately 0.1% of the total viable count (Holdeman et al., 1977). Like *G. haemolysans*, it has also been found in the intestinal contents of pigs (Molitoris et al., 1986).

Little is known about the distribution of the more recently described *Gemella* species. Both *G. bergeri* and *G. sanguinis* have been isolated from human clinical sources, including from blood cultures of patients with subacute bacterial endocarditis (Collins et al., 1998a; Collins et al., 1998b). The only known source of *Gemella palaticanis* is the oral cavity of a dog (Collins et al., 1999), whereas *G. cuniculi* was originally recovered from a submandibular abscess of a rabbit (Hoyles et al., 2000).
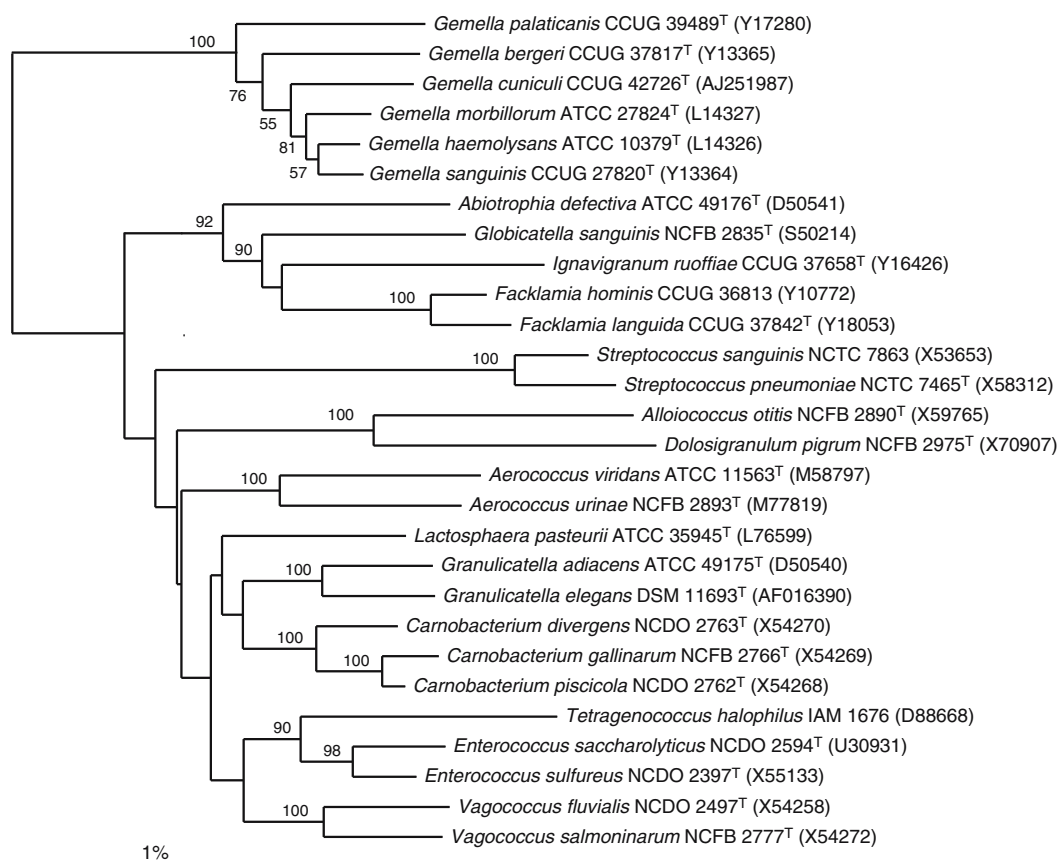
Fig. 1. Dendrogram showing the phylogenetic interrelationships of *Gemella* species based on 16S rRNA gene sequences. The tree was constructed using the neighbor-joining method. Bootstrap re-sampling values, expressed as percentages of 500 replications, are given at the branching points.

Isolation and Maintenance

Gemella species can be grown on blood agar. With the exception of *G. morbillorum*, *Gemella* species should be incubated in the presence of oxygen (with or without additional CO_2), but for the growth of *G. morbillorum*, oxygen should be excluded (Berger and Pervanidis, 1986). While incubation in elevated CO_2 concentrations stimulates the growth of gemellae, the aerotolerant *G. morbillorum* prefers an anaerobic atmosphere.

For isolating *G. haemolysans* from oropharyngeal swabs, organisms should be streaked onto blood agar, where they form smooth, non-hemolytic or α-hemolytic colonies that resemble viridans streptococci. The hemolytic reaction, an important characteristic in the presumptive identification of *G. haemolysans*, depends on the nature of the growth medium (choice of blood and agar base). β-Hemolysis is only consistent on Mueller-Hinton agar supplemented with rabbit blood. This is the best-suited medium for the isolation of colonies suspected of being *G. haemolysans*. For isolating *G. haemolysans* from septicemic infections, freshly drawn blood can be transferred to commercially available blood culture media, and incubated aerobically or anaerobically. Generally, the liquid medium becomes slightly turbid after 3 days, after which culture fluid can be streaked onto blood (rabbit, sheep or horse) agar plates. The isolation of *G. haemolysans* from cerebrospinal fluid (CSF) can be achieved by streaking out a small amount of CSF deposit onto blood agar (Mitchell and Teddy, 1985). *Gemella haemolysans* has also been recovered from dental plaque (see de Jong and van der Hoeven [1987] for a detailed description of the method).

Gemella morbillorum has been recovered from a broader range of clinical sources than *G. haemolysans*. The organism can be isolated from swabs and pus by plating onto blood (usually 5% defibrinated sheep blood) agar plates. The minute colonies of are generally either non-hemolytic or are surrounded by a zone of α-hemolysis (greening; Holdeman and Moore, 1974; Facklam, 1977). Hemolysis may or may not be influenced by blood source (Facklam and Wilkinson, 1981; Berger and Pervanidis, 1986). Hemolysis may also be influenced by the strain, presence/absence of oxygen and CO_2 levels. In *G. morbillorum*, hemolysis is not a constant character. *Gemella morbillorum* can be recovered from dental plaque; samples are transferred to thioglycolate broth (Difco) containing 20% beef infusion, and dilutions streaked onto Columbia blood agar incubated in an atmosphere containing H_2, CO_2 and N_2 (1:1:8; Kolenbrander and Williams, 1983). *Gemella morbillorum* has been

isolated from the human intestinal tract using rumen-fluid-glucose-cellobiose agar (RGCA) used for culturing anaerobes (Moore and Holdeman, 1974; Holdeman et al., 1976).

During laboratory work, gemellae can be routinely maintained on a variety of agar media, such as heart infusion agar, Mueller-Hinton agar (Difco, BBL), trypticase soy agar (BBL), and Columbia agar (Biomerieux), supplemented with blood (5–7%), serum (5–10%) or ascitic fluid (10–20%). Plates can be incubated aerobically (with or without additional CO_2). Newly isolated *G. morbillorum* strains, which are not yet adapted to these conditions, should be cultured anaerobically. The growth of *G. morbillorum* may be improved by the addition of 0.001% pyridoxal-HCl to the blood agar. Heart infusion, brain heart infusion, and trypticase soy broths enriched with serum or ascitic fluid serve as suitable liquid media. For *G. morbillorum*, Todd-Hewitt broth supplemented with 0.001% pyridoxal-HCl is a good medium, as is thioglycolate broth supplemented with 10% serum for *G. haemolysans*.

For short-term preservation, *G. haemolysans* can be streaked onto heart infusion agar slants with 10% ascitic fluid or 10% inactivated serum added, or stabbed into the medium, incubated for 1 day, and then kept at room temperature (Berger, 1992). *Gemella morbillorum* has been preserved in chopped meat-glucose broth at room temperature (Kannangara et al., 1981). *Gemella morbillorum* has been maintained on trypticase soy agar containing 5% sheep blood (Dzink et al., 1984) in an atmosphere of N_2, H_2 and CO_2 (8:1:1).

For long-term preservation, *Gemella* strains can be maintained in media containing 15–20% glycerol stored at –70°C, or lyophilized.

Identification

The identification of *Gemella* can be achieved using either morphological and biochemical criteria or by molecular taxonomic methods. All 6 species possess characteristic 16S rRNA gene sequences, and sequence analysis is probably the quickest and most reliable tool for speciation (Collins et al., 1999; Hoyles et al., 2000). However, identification to the species level in routine laboratories can also be achieved by phenotypic methods.

On blood-based agars, gemellae grow slowly forming small, circular, entire, low convex, translucent to opaque, smooth (occasionally mucoidal) colonies that somewhat resemble viridans streptococci. Some strains of gemellae are hemolytic (Berger, 1992). On trypticase soy sheep blood agar, most strains are α- or non-

hemolytic. The expression of hemolysis can depend on the type of blood and agar base used. Wide zone (β) hemolysis may be observed with some strains, especially *G. haemolysans*, on blood agar bases containing rabbit blood. *Gemella morbillorum* produces greening or fails to hemolyze the blood, although exceptional strains of this species may display wide zone hemolysis. Examination for β-hemolysis is best performed on Mueller-Hinton agar supplemented with rabbit blood (Berger, 1992).

Cellular morphology can be helpful in differentiating gemellae from streptococci. Gemellae divide in two planes, generally at right angles to each other. Cells are cocci arranged in pairs, often with adjacent sides flattened, or arranged in tetrads, small irregular clusters or short chains. Pleomorphism may be observed. Morphology varies with strain and cultural conditions (Berger, 1992). Size of the cells may vary considerably; diameter varies from about 0.5 μm to more than 1 μm and "giant cells" have been observed (Berger, 1992). In *G. morbillorum*, pleomorphism may be very pronounced, coccal forms are frequently elongate, and cells may be of unequal size. Elongate cells are generally 0.5 by 1.2 μm (Holdeman and Moore, 1974), but longer cells (up to 2–3 μm) have been reported (Prévot, 1933). Gemellae possess a Gram-positive cell wall type but may appear Gram-variable owing to their relatively rapid decolorization with alcohol. Though of the Gram-positive type, their walls are relatively thin (10–20 nm; Reyn et al., 1970; Mills et al., 1984), which probably accounts for their Gram-variable character. Electron microscopic studies have revealed the cells of *G. haemolysans* are surrounded by a "corona" of floccular or fibrous material arranged radially at the cell surface (Reyn et al., 1966; Reyn et al., 1970). An outer layer of similar structure has also been observed in *G. morbillorum* (Mills et al., 1984). It is thought that the fibrous material in *G. morbillorum* is a polyanionic polysaccharide, which is extensively cross-linked.

The optimum growth temperature of gemellae is probably 35–37°C, but species can grow over a wider range of temperatures. They do not grow at 10 or 45°C or in broth containing 6.5% NaCl. They are bile esculin negative. Gemellae are catalase and oxidase negative; they are fermentative, producing acid from glucose and some other carbohydrates. The major end products of anaerobic glucose metabolism by *G. haemolysins* and *G. morbillorum* are L-lactic and acetic acids (Brooks et al., 1971; Holdeman and Moore, 1974; Stackebrandt et al., 1982); formic acid is also detected in both species. Under aerobic conditions, the end products of glucose metabolism formed by *G. haemolysans* are equimolar amounts of acetate and CO_2 (Stackebrandt et al., 1982). The presence of fructose-1,6-bisphosphate aldolase indicates that *G. haemolysans* degrades glucose by the Embden-Meyerhof pathway (Stackebrandt et al., 1982). Acid formation in carbohydrate broth by *Gemella* species, using conventional tests, can be somewhat varied with some substrates. Acid is produced from mannitol and sorbitol by some *Gemella* strains, but these can be variable characteristics depending on the test method. Using the API rapid ID 32Strep system, *G. sanguinis*, *G. cuniculi* and most strains of *G. morbillorum* ferment these substrates, whereas *G. haemolysans* and *G. palaticanis* do not. Some strains of *G. bergeri* produce acid from mannitol but fail to ferment sorbitol. *Gemella haemolysans*, *G. morbillorum*, *G. palaticanis* and *G. sanguinis* produce acid from maltose and sucrose, whereas *G. bergeri* and *G. cuniculci* do not. *Gemella palaticanis* produces acid from lactose, whereas other gemellae fail to ferment this substrate. Using the same API test system, none of the *Gemella* species produce acid from D-arabitol, L-arabinose, cyclodextrin, glycerol, melibiose, melezitose, methyl-β-D-glucopyranoside, pullulan, raffinose, ribose or tagatose. Gemellae do not hydrolyze esculin, gelatin, hippurate or urea. They do not degrade casein or liquefy coagulated serum. None of the *Gemella* species reduce nitrate. However the reduction of nitrite appears to be a regular characteristic of *G. haemolysans* but not of *G. morbillorum* (Berger, 1992). It is not known whether the other four *Gemella* species reduce nitrite. Some species produce acid and alkaline phosphatases. Activity for both of these enzymes, are detected in *G. cuniculi*, *G. haemolysans* and *G. sanguinis* using the API rapid ID 32Strep and API ZYM systems, but not in the other species. Most *Gemella* strains are pyrrolidonyl arylamidase positive; the only known isolate of *G. cuniculi* is pyrrolidonyl arylamidase negative. All gemellae are arginine dihydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase, α-fucosidase, lipase C14, trypsin, *N*-acetyl-β-glucosaminidase, and valine arylamidase negative.

Numerous biochemical tests are potentially useful in differentiating the various *Gemella* species, but some of these vary according to the test method used. There is sometimes poor correlation between results obtained by conventional methods and miniaturized test systems (such as API kits). *Gemella haemolysans* can however usually be distinguished from *G. morbillorum* by producing alkaline phosphatase and by failing to produce acid from mannitol and sorbitol. Most strains of *G. morbillorum* are alkaline phosphatase negative, and most produce acid from mannitol and sorbitol (Berger, 1961; Berger and Pervanidis, 1986). *Gemella sanguinis* and *G.*

Table 1. Characteristics useful in distinguishing species of the genus *Gemella*.

	G. bergeri	*G. cuniculi*	*G. haemolysans*	*G. morbillorum*	*G. palaticanis*	*G. sanguinis*
Acid from:						
Lactose	−	−	−	−	+	−
Mannitol	d	+	−	d	−	+
Maltose	−	−	+	+	+	+
Sorbitol	−	+	−	d	−	+
Sucrose	−	−	+	+	+	+
Trehalose	−	−	−	−	+	−
Production of:						
PAC	−	+	+	−	−	+
PAL	−	+	+	−	V	+
APPA	d	−	−	d	+	+
GTA	−	−	d	−	+	−
VP test	−	−	−	−	−	+

Symbols: +, present in all strains; −, absent in all strains; d, present in some strains; and V, variable.
Abbreviations: PAC, acid phosphatase; PAL, alkaline phosphatase; APPA, alanyl phenylalanine proline arylamidase; GTA, glycyl tryptophan arylamidase; and VP, Voges-Proskauer.
Tests performed using API rapid ID32 Strep system except for production of PAC, which is performed using API ZYM kit.

bergeri, also associated with human clinical sources, can be readily distinguished from each other and the aforementioned species biochemically using the API rapid ID 32Strep system. *Gemella sanguinis* is similar to *G. morbillorum* in forming acid from mannitol and sorbitol but differs from the latter species in producing alkaline phosphatase. Similarly, *G. bergeri* differs from *G. haemolysans*, *G. morbillorum* and *G. sanguinis* by failing to produce acid from maltose and sucrose (Collins et al., 1998b). It further differs from *G. haemolysans* and *G. sanguinis* by being alkaline phosphatase negative (Collins et al., 1998a). Tests useful in distinguishing between the various *Gemella* species, using the API rapid ID 32Strep system, are shown in Table 1.

The DNA G+C content of *G. haemolysans*, *G. bergeri*, *G. morbillorum*, *G. palaticanis* and *G. sanguinis* are 33.5 ± 1.6 (by the buoyant density [Bd] method), 32.5 (by the melting temperature [T_m] method), 30 (T_m), 32 (T_m), and 31 (T_m) mol%, respectively. The cell wall murein of *G. morbillorum* is of the L-Lys-Ala$_{1-3}$ type (Kilpper-Bälz and Schleifer, 1988). There is no information on the murein composition of the other *Gemella* species.

Antimicrobial Susceptibility

Knowledge of the antimicrobial susceptibilities of species of the genus *Gemella* is somewhat fragmentary (Buu-Hoi et al., 1982; Berger, 1992). Both *G. haemolysans* and *G. morbillorum* are highly sensitive to penicillins, cephalosporins, tetracyclines, chloramphenicol and lincomycins. *Gemella haemolysans* is strongly inhibited by macrolide antibiotics (erythromycin, spiramycin and oleandomycin), vancomycin, ristocetin, novobiocin and tyrothricin. Some strains are however resistant to erythromycin and tetracycline. A majority of strains are also inhibited by bacitracin and fusidic acid. *Gemella haemolysans* is resistant to sulfonamides and trimethoprim and also displays low-level resistance to aminoglycosides (streptomycin, kanamycin, gentamycin, tobramycin, amikacin and neomycin). Synergy between penicillin G and either gentamycin or streptomycin, and between vancomycin and the aforementioned aminoglycosides is also observed (Buu-Hoi et al., 1982). The antimicrobial susceptibility patterns of *G. haemolysans* and *G. morbillorum* are typical of Gram-positive organisms and resemble those of viridans streptococci. *Gemella bergeri* and *G. sanguinis* are sensitive to vancomycin, but their susceptibilities to other antimicrobials remain unknown. There is no information on the susceptibilities of *G. palaticanis* and *G. cuniculi* to antibiotics.

Clinical Significance

Gemella haemolysans and *G. morbillorum*, like many other commensal bacteria of the human microbiota, are opportunistic pathogens. Both species are capable of causing severe localized and generalized infection, particularly in immunocompromised patients (Durak et al., 1983; Etienne et al., 1984; Mitchell and Teddy, 1985; Eggelmeier et al., 1992; Petit et al., 1993; Pradeep et al., 1997).

Gemella haemolysans has been isolated from blood cultures of patients with endocarditis (Buu-Hoi et al., 1982; Chatelain et al., 1982; Blin et al., 1984; Laudat et al., 1984; Kaufhold et al., 1989; Brack et al., 1991; Morea et al., 1991; Fresard et al., 1993). The organism has also

been isolated from CSF cultures of patients with meningitis (Mitchell and Teddy, 1985; Aspevall et al., 1991; May et al., 1993; Petit et al., 1993).

Gemella morbillorum are infrequent clinical isolates, but nevertheless it is clear that this species can participate in serious human infections. Owing to the regular recovery of *G. morbillorum* from the blood and throat of measles patients, this organism was mistakenly believed to be the causative agent of the disease (Tunnicliff, 1917; Tunnicliff, 1933; Duval and Hibbard, 1927). Prévot et al. (1967) reported *G. morbillorum* from various suppurative processes, including dental abscess, tonsillitis, axillary hydroadenitis, pleuritis, lung abscess, peritonitis, subphrenic abscess, and pyelonephritis. Facklam (1977) recovered *G. morbillorum* from blood, respiratory, genitourinary, wound and abscess (including one isolate from brain abscess) specimens. In his study of over 1200 viridans streptococci from clinical sources, *G. morbillorum* constituted more than 3% of the strains. *Gemella morbillorum* has since been recovered from blood cultures of patients with endocarditis (e.g., Durak et al., 1983; Etienne et al., 1984; Coto and Berk, 1984; Maxwell, 1989; Omran and Wood, 1993) and from a variety of other sources such as from cultures of synovial fluid from septic arthritis (Von Essen et al., 1993), from CSF cultures from patients with meningitis (Debast et al., 1993) or brain abscess (Murray et al., 1998).

There is very little information about the possible clinical significance of other *Gemella* species. Both *G. bergeri* and *G. sanguinis* have been isolated from human clinical specimens including from blood cultures of persons with bacterial endocarditis (Collins et al., 1998b; Collins et al., 1998a; Shukla et al., 2002). It is possible that *G. bergeri* and *G. sanguinis* are responsible for a similar range of conditions as those due to *G. haemolysans* and *G. morbillorum*. Indeed owing to difficulties encountered in identifying *Gemella* species and limitations in tests often used in routine laboratories, it seems likely that these organisms may have been misidentified as *G. haemolysans* and/or *G. morbillorum* in the past. Of the remaining *Gemella* species, *G. palaticanis* was originally isolated from the oral cavity of a dog (Collins et al., 1999), whereas the only known strain of *G. cuniculi* was recovered, in mixed culture, from a submandibular abscess of a rabbit (Hoyles et al., 2000). There is currently no evidence that these species cause disease.

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CHAPTER 1.2.15

The Genus *Kurthia*

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Introduction

In 1883, H. Kurth published the description of *Bacterium zopfii*, which he had isolated from the intestinal contents of chickens (Kurth, 1883). Two years later, Trevisan (1885) created the genus *Kurthia* with *K. zopfii* as the type species. However, over the ensuing years Kurth's organism was given a variety of generic names, including *Zopfius* (Wenner and Rettger, 1919); the valid name *Kurthia* really came into general use only after the publication of the seventh edition of *Bergey's Manual of Determinative Bacteriology* (Breed et al., 1957). The genus *Kurthia* contains three species, *K. zopfii*, *K. gibsonii* (Keddie and Shaw, 1986), and *K. sibirica* (Belikova et al., 1986). Additional species are mentioned in the literature but have not been validated, e.g., "*Kurthia catenaforma*" (Kato et al., 1972), "*Kurthia bessonii*" (Pancoast et al., 1979) or "*Kurthia variabilis*" (Severi, 1946).

Reference strains are *K. zopfii* DSM {20580}^{T} (NCIB {9878}^{T}, NCTC {10597}^{T}); *K. gibsonii* DSM {20636}^{T} (NCIB {9858}^{T}, ATCC 43195^{T}); *K. sibirica* DSM {4747}^{T} (VKM {V-1549}^{T}, CCM {3477}^{T}).

Kurth isolated the organism now called "*K. zopfii*" by streaking material from the intestinal contents of chickens onto nutrient gelatin plates. When the plates were incubated, the organism grew out from the original streak and through the gelatin in long, fine, apparently branched threads. For a number of years following the original isolation, *K. zopfii* aroused considerable interest because of its characteristic growth patterns in nutrient gelatin, and various attempts were made to explain the observed phenomena. Most characteristic is the appearance in a gelatin slant. If the slant is inoculated with a single central streak and incubated in the near vertical position, then the resultant growth resembles a bird's feather (Boyce and Evans, 1893; Zikes, 1903; Sergent, 1906; Sergent, 1907; Jacobsen, 1907; Kufferath, 1911). The outgrowths, which appear to follow the lines of stress in the gelatin, are presumably a result of the organism's marked filament-forming ability coupled with an inability to hydrolyze gelatin; both motile and nonmotile strains exhibit this phenomenon (Keddie, 1949).

In the few decades following its original isolation, *K. zopfii* (or *K. zenkeri*, a synonym of *K. zopfii*) was isolated from a variety of sources, including fresh and putrefied meat (Günther, 1896; Jacobsen, 1907; Wenner and Rettger, 1919), wastewater and air from abattoirs (Jacobsen, 1907), preserved sausage (Günther, 1896), and pus from a cat's ear (Boyce and Evans, 1893). It was also reported to occur in feces (Flügge, 1896), water (Flügge, 1896; Migula, 1900), and to be common in milk (Orla Jensen, 1921). One strain of an organism considered to be *K. zopfii* was isolated from air at an altitude exceeding 3,000 m (10,000 ft) (Proctor, 1935). A nonvalidated strain of "*Kurthia bessonii*" has been isolated from a variety of clinical materials (Liebschers et al., 1965; Pancoast et al., 1979), but there is no evidence of pathogenicity in authentic members of the genus.

Following this early interest in *K. zopfii*, the genus *Kurthia* received scant attention for many years, but the more recent rediscovery that *K. zopfii* and the organism now called "*K. gibsonii*" frequently occur as components of the aerobic flora of meats and meat products once again focused attention on the genus. However, bacteria of the genus *Kurthia* do not appear to have been implicated directly in the spoilage process of meats and meat products.

Bacteria of the genus *Kurthia* are strictly aerobic, Gram-positive rods that do not produce acid from glucose. Consequently, in earlier studies they were sometimes considered to be taxonomically related to one or more of the aerobic genera of coryneform bacteria. Indeed, some earlier numerical taxonomic surveys seemed to support this view (see Shaw and Keddie, 1983a). The tentative inclusion of *Kurthia* in the section on the "Coryneform Group of Bacteria" in the eighth edition of *Bergey's Manual of Determinative Bacteriology* was interpreted as supporting this similarity (e.g., see Ludwig et al., 1981), and presumably led to the inclusion of the chapter on "The Genus *Kurthia*" in the section on "The Coryneform Bacteria" in the first edition of "*The Prokaryotes*" (Starr et al., 1981). However,

chemical studies of the peptidoglycan structure (Schleifer and Kandler, 1972), isoprenoid quinone composition (Collins et al., 1979), polar lipid composition (Goodfellow et al., 1980), and the G+C content of the DNA (Belikova et al., 1980; Belikova et al., 1986; Shaw and Keddie, 1984) distinguish *Kurthia* from members of the "coryneform group."

Phylogenetic Position

The results of early rRNA oligonucleotide sequencing studies of *Kurthia zopfii* ATCC 6900 and various Gram-positive bacteria confirm that *Kurthia* is only very distantly related to the aerobic coryneform bacteria and is more closely related to members of the genera *Bacillus*, *Lactobacillus*, *Staphylococcus* and *Streptococcus* (Ludwig et al., 1981). This taxonomic placement was supported by the results of a numerical taxonomic study (Shaw and Keddie, 1983a), which showed a close phenotypic similarity between *Kurthia* and some aerobic *Bacillus* spp. but little similarity with various genera of aerobic coryneform bacteria.

Analyses of low molecular weight RNA profiles of various bacteria, *Kurthia*, and related Gram-positive organisms with a low DNA G+C content (Collins-Thompson et al., 1991) demonstrated that the pattern of *K. zopfii* and *K. gibsonii* were identical and similar to those of members of *Brochothrix* and *Listeria*. However, bacilli were not included in that study.

From 1984 on, the phylogenetic position of *Kurthia* and certain other nonsporeforming aerobic spherical and rod-shaped Gram-positive organisms was fully elucidated on the basis of rRNA cataloguing but mainly of almost complete 16S rRNA gene sequences. Unexpectedly, members of *Filibacter* (Clausen et al., 1985), *Sporosarcina*, *Planococcus* and *Marinococcus* (Farrow et al., 1992), as well as *Kurthia*, *Caryophanon*, *Planococcus* and *Exiguobacterium* (Farrow et al., 1994) were found to be closely related to members of the genus *Bacillus* (Stackebrandt et al., 1987; Ash et al., 1991; Stackebrandt and Swiderski, 2002). The genus *Bacillus* itself was found to contain several remotely related phylogenetic branches, many of which are today described as individual genera, including among others *Paenibacillus* (Ash et al., 1993), *Alicyclobacillus* (Wisotzkey et al., 1992), *Alicyclobacillus* and *Brevibacillus* (Shida et al., 1996), *Virgibacillus* (Heyndrickx et al., 1998) and *Geobacillus* (Nazina et al., 2001). The core genus of *Bacillus* still forms a phylogenetically broad cluster, consisting of different groups (Ash et al., 1991), one of which, the *Bacillus* group 2, embraces the round spore-forming bacilli (e.g.,

B. sphaericus, *B. insolitus*, and *B. silvestris*) and their nonsporeforming relatives (Stackebrandt et al., 1987).

The type strains of the three *Kurthia* species form a tight phylogenetic cluster sharing 95.8–97.7% 16S rRNA gene sequence similarity (Fig. 1). The branching is supported by high bootstrap values. *Kurthia* species are closely related to the type strains of two *Bacillus* species, i.e., *B. pycnus* JCM 11075[T] and *B. neidei* JCM 11077[T] (94.2–96.2%). The low bootstrap values indicate that the stability of their branching is less significant and may change with the inclusion of other sequences in the database. These five species form a sister clade of a cluster consisting of species of *Bacillus*, *Sporosarcina*, *Filibacter* and *Planococcus*. The hallmark of cluster 2 is the presence of either L-lysine or ornithine at position 3 of the peptide side chain of peptidoglycan. D-Glutamic acid or D-aspartic acid form the interpeptide bridge (Fig. 1). This composition is significantly different from the amino acid composition of the type species *Bacillus subtilis* and its many relatives, which contain a directly linked peptidoglycan containing *meso*-diaminopimelic acid as the diagnostic amino acid. It thus appears that the formation of round spores or loss of spores, differences in cell wall composition, and morphological variations are reflections of an individual evolutionary path of organisms of *Bacillus* group 2 that may lead to the recognition of one or more new genera, depending on the taxonomic evaluation of these differences (Rheims et al., 1999).

Genes other than those coding for 16S rRNA have rarely been sequenced. An example is the suite of eleven biotin biosynthesis genes involved in the stepwise synthesis of biotin from an unknown precursor via the pimeloyl-CoA pathway in *Kurthia* sp. DSM 10609 (Kiyasu et al., 2001; accession numbers [{www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=AB045873}{AB045873}], [{www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=AB045873}{AB045873}], and [{www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=AB045875}{AB045875}]). The lack of the *bioW* gene, coding the pimeloyl-CoA synthetase suggest that unlike in *Bacillus subtilis* and *B. sphaericus*, pimelic acid does not serve as the precursor; the presence of the *bioC* and *bioH* genes makes it likely that a modified pathway via acetyl-CoA is used, similar to the one found in *Escherichia coli*.

Besides a chitinase gene of *K. zopfii* (accession number [{www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=D63702}{D63702}]), the *cpn60* (chaperonin 60) gene has been partially sequenced for all three type strains of *Kurthia* (a123713, a123714 and a123716). This

Fig. 1. Neighbor-joining tree (Felsenstein, 1993) generated on the basis of 16S rRNA gene sequences showing the nearest neighbors of members of the genus *Kurthia*. Bootstrap value was calculated for 500 replicate trees. The bar corresponds to a 1% difference in nucleotide sequences.


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        100  Bacillus sphaericus DSM 28 (AJ310084)
             Bacillus fusiformis DSM 2898 (AJ310083)
   100
             Caryophanon latum DSM 14151 (AJ491302)
        89
             Caryophanon tenue DSM 14152 (AJ491303)
             Bacillus silvestris DSM 12223 (AJ006086)
             Kurthia sibirica DSM 4747 (AJ605774)
        87
          86 Kurthia zopfii NCIMB 9878 (X70321)
             Kurthia gibsonii NCIMB 9758 (X70320)
             Bacillus pycmus JCM 11075 (AF169531)
             Bacillus neidei JCM 11077 (AF169520)
             Sporosarcina macmurdoensis CMS 21w (AJ514408)
             100  Bacillus psychrodurans DSM 11713 (AJ277984)
        100       Bacillus psychrodurans DSM 11706 (AJ277983)
             Bacillus insolitus DSM 5 (X60642)
             Filibacter limicola DSM 13886 (AJ292316)
        100  Planococcus psychrophilus CMS53or (AJ314746)
             Planococcus citreus NCIMB 1493 (X62172)
             Bacillus subtilis subsp. subtilis DSM 10 (AJ276351)
                      0.01
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gene has the potential to serve as phylogenetic marker (Roger et al., 1998) as well as a potentially universal target for identification (Goh et al., 2000).

Habitats

There have been few systematic studies of the occurrence of *Kurthia* spp. in natural materials. However, from the limited information available, it seems that the sources from which authentic *Kurthia* spp. are regularly isolated are meat and meat products and animal feces that have lain on soil, straw, etc., for a short time. *Kurthia zopfii* and *K. gibsonii* are associated with off-flavors and taints of fresh and cured meat products stored at elevated temperatures, and the psychrophilic *K. sibirica* is associated with refrigerated samples (Keddie and Jones, 1992).

What appeared to be the most unusual source was reported by Belikova et al. (1980), who isolated several strains of *Kurthia* from samples of the intestinal contents and stomach of the Magadan (Susuman) mammoth found preserved in the permafrost in East Siberia. Of 13 strains referred to by these authors as *K. zopfii*, four had the characters of that species, three appeared to be *K. gibsonii* and the remainder were psychrophilic strains that differed from both these species (see Keddie and Shaw, 1986). A new species,

K. sibirica, was created for the psychrophilic strains (Belikova et al., 1986). While we obviously cannot comment on the ability of *Kurthia* to survive for several thousand years in permafrost, we are nevertheless certain that the highly aerobic *Kurthia* would be unable to multiply in the highly anaerobic environment of the animal gut. A solution to the problem became apparent when it was revealed that after the mammoth was removed from the permafrost it was kept in an unfrozen state for several days before examination (Belikova et al., 1986). Presumably the carcass became contaminated with *Kurthia* which then multiplied during storage. Indeed, more recent studies (see above) strongly suggest that Kurth's original isolates came from chicken intestines that had been stored at room temperature for a few days; like the mammoth, contamination with *Kurthia* (possibly from flies) would have been followed by multiplication during storage.

Meat and Meat Products

Keddie (1949) isolated *K. zopfii* and the organism now called "*K. gibsonii*" from a variety of samples of fresh meat, fat, etc., and from meat that was allowed to putrefy at room temperature. These organisms were also readily isolated from meat, fat, wastewater, etc. from an abattoir, thus confirming the early report of Jacobsen (1907).

According to Holzapfel (1992), saprophytic Gram-positive bacteria, e.g., *K. zopfii* and *K. gibsonii*, are probable minor groups belonging to the initial microbial population on the surface of meat (from healthy animals) kept in the abbatoir environment. Among other Gram-positive bacteria, *Kurthia* spp may be involved in spoilage of fresh and processed meat (Gardner, 1969; Keddie, 1981).

Ingram (1952) found that Gram-positive, non-sporeforming rods constituted about 10% of the aerobic flora of internally tainted, cured pork legs. Some of these rods were considered to be *Kurthia* spp. Also, *Kurthia zopfii* and *K. gibsonii* have been isolated from pork and pork products (Gardner et al., 1967b; Shaw and Keddie, 1983a), various ground fresh meat products (Gardner, 1969; Shaw and Keddie, 1983a), from an eviscerated, polyethylene-wrapped chicken stored at 15°C until off-flavors developed, from an irradiated lamb carcass stored for about 7 weeks at 1°C (quoted by Gardner, 1969), and from spoiled British sausages (Dowdell and Board, 1971).

Information on the numbers of *Kurthia* in these various meat products is sparse. Gardner (1969) noted that although members of the genus were regularly isolated from fresh, comminuted meat products, they usually accounted for only up to 10% of the total aerobic count. On the other hand, in six samples of pork stored at 16°C for 5 days in gas-impermeable film, gas-permeable film, or with no film, *Kurthia* represented 12–44, 9–76, and 5–69%, respectively, of the total aerobic flora but were not detected in similar samples stored at 2°C for 14 days (Gardner et al., 1967b). Holzapfel (1992) indicated growth and survival parameters for temperature between 5 and 45°C, for pH between 5.0 and 8.5, and for a_w of ca. 0.95. From these and other observations (Gardner and Carson, 1967a), Gardner (1969) concluded that *Kurthia* could compete favorably with other aerobic spoilage bacteria in meat stored at about 16°C, but not in meat stored at refrigeration temperatures. The two strains isolated by D'Aubert et al. (1975) from refrigerated, vacuum-packed meat and identified by them as *Kurthia* spp. did not have the characteristics of the genus.

Animal Feces in Contact with Soil, Straw, and Other Materials

In our experience, *Kurthia* can regularly be isolated from the feces of certain domestic animals provided it has lain on soil, straw, etc., for a short time. Keddie (1949) was unable to isolate *Kurthia* from the cecal contents of chickens or from the freshly voided feces, but three of nine samples of chicken feces that had lain on the soil for some time yielded the organism. *Kurthia zopfii* and *K. gibsonii* have been isolated from many different samples of soil-contaminated chicken feces and from various situations in poultry houses (Shaw and Keddie, 1983a). Strivastava et al. (1972) also reported the isolation of two presumptive *Kurthia* strains from a poultry house and a hatchery. Also, pig feces contaminated with straw or soil have yielded *K. zopfii* and *K. gibsonii*, as have similarly contaminated feces of horses and cows (Shaw and Keddie, 1983a).

Clinical Sources

Several strains of presumptive *Kurthia* spp. have been isolated from various clinical sources and most frequently from the feces of patients suffering from diarrhea. Some organisms were considered to be "*K. bessonii*" or "*K. variabilis*," neither of which was included in The Approved Lists of Bacterial Names (Skerman et al., 1980) and are therefore not legitimate species.

Frequently, the descriptions of the isolates mentioned contain apparently contradictory statements (e.g., facultative anaerobes; do not ferment sugars) or are too limited to be able to judge whether the organisms were indeed authentic kurthias.

Severi (1946) isolated what he considered to be a new species, "*K. variabilis*," from feces in a case of mild food poisoning. Elston (1961) isolated three strains identified as "*K. bessonii*" from a pilonidal cyst, sputum, and a diarrheal stool, and Faoagali (1974) isolated a presumptive *Kurthia* sp. from a routine eye swab. Jarumilinta et al. (1976) isolated presumptive *Kurthia* spp. from the upper intestinal tract of six of 25 patients suffering from acute diarrhea, but only from one of 24 control patients. More recently Yang et al. (1985) isolated an organism they identified as *K. zopfii* from the blood of an infant with septicemia. However, in all of these cases, the connection between the occurrence of the presumptive *Kurthia* spp. and the clinical condition was, at most, tenuous.

Other Sources

The isolation of presumptive *Kurthia* spp. has been reported from sources as diverse as "sloughing spoilage" of ripe olives ("*K. bessonii*"; Patel and Vaughn, 1973), the gut of a crab ("*K. variabilis*"; Saha and Raychaudhuri, 1973), wet-stored wood (Berndt and Liese, 1973), and from dental plaque of beagle dogs (*Kurthia*?; Wunder et al. 1976); but like those from clinical sources, the accuracy of identification of many of these isolates is doubtful.

However, more recent evidence supports the early statement by Orla Jensen (1921) that *K. zopfii* may occur in milk because this species was found at levels of about 10^4/ml in some samples of bulked, cold-stored (7°C) raw milks (C. M. Cousins, personal communication). The spoilage effect of *Kurthia* strains on fresh milk and fresh vegetables must be considered with skepticism (Gardner, 1969). *Kurthia zopfii* has occasionally been isolated from soil and surface waters (Shaw and Keddie, 1983a) and a few observations indicate that organisms resembling *Kurthia* but distinct from the accepted species occur in certain peats, namely, low-moor peat (Janota-Bassalik, 1963) and Antarctic peat (Baker and Smith, 1972).

To our knowledge *Kurthia* spp. have not yet been exploited in any biotechnological process.

Isolation

Kurthia zopfii and *K. gibsonii* may be isolated by using methods that exploit their unusual cultural properties. One of the most successful is a gelatin streak method similar to that used by Kurth and the early investigators (Keddie, 1949). However, a simple agar streak technique is a useful additional method (see Keddie, 1981).

These methods were not used for isolation of *K. sibirica*, but the report that strains of this species produce a "bird's feather" growth on nutrient gelatin (Belikova et al., 1986) suggests that they might be successful if a suitably low incubation temperature is chosen.

Gelatin Streak Method for Isolating *Kurthia* (Keddie, 1949)

A nutrient gelatin medium (YNG) of the following composition is used.

YNG Medium

Meat extract (Lab-Lemco powder, Oxoid)	4 g
Peptone (Difco)	5 g
Yeast extract (Difco)	2.5 g
NaCl	5 g
Gelatin (BDH)	60 g

Dissolve ingredients in distilled water and dilute up to 1 liter. Adjust pH to 7.0. For quantities up to 100 ml, sterilize at 115°C for 30 min. Pour plates with about 20 ml of molten YNG medium and allow to solidify in the refrigerator. Inoculate plates heavily with a single central streak of the material to be examined (or with a suspension or macerate of solid material in a small amount of sterile water). Incubate plates at 20°C with the lids uppermost and examine daily. The gelatin is usually soon liquefied around the streak, but in successful cultures, filamentous

outgrowths appear beyond this zone in 2–3 days, and after 3–4 days, a tangled mass of filaments may completely permeate the solid part of the medium if it is not completely liquefied. To obtain a pure culture, streak a small piece of gelatin containing outgrowths on yeast extract-nutrient agar medium (YNA). YNA is similar to YNG but is solidified with agar instead of gelatin.

The composition of the nutrient gelatin medium used is important, and the concentration and brand of gelatin used is particularly important; not all brands allow the typical outgrowths. The medium should be inoculated with a reference strain of *K. zopfii* (NCIB 9878) to test its ability to allow good outgrowth production. The gelatin manufactured by the BDH Chemical Co., Poole, England, is satisfactory but with some batches a higher concentration than that stated (up to 100 g/liter) may be required. Although all *Kurthia* strains tested grow well in YNB (i.e., YNG medium without gelatin), medium YNG prepared with some batches of gelatin has given poor growth. Dissolving the constituents of YNG in mineral base E (Owens and Keddie, 1969) to give MYNG (Shaw and Keddie, 1983a) overcomes this problem. When MYNG is autoclaved, a precipitate (which should be dispersed before pouring plates) is produced; the precipitate does not interfere with isolation. Overgrowth with fungi may be a problem with some materials. This can be prevented by adding nystatin (Squibb) to the molten YNG to a concentration of 10 units/ml before pouring plates.

The above-mentioned isolation procedure is not strictly selective and one should watch for filamentous or feathery growth in gelatin media, of "medusa" head appearance of young colonies under low magnification, and rhizoid growth or granular appearance on YNA (Keddie and Jones, 1992). Other bacteria that may produce outgrowths in gelatin similar to those of *Kurthia* are certain chain-forming *Bacillus* species, particularly *B. cereus* subsp. *mycoides*, although they may liquefy gelatin.

The agar streak method may be used in addition to that described above and can give successful results when rapid liquefaction of gelatin has prevented isolation of *K. zopfii* by the gelatin streak method.

Agar Streak Method for Isolating *Kurthia* (see Keddie, 1981)

The YNA Medium

Meat extract (Lab-Lemco powder, Oxoid)	4 g
Peptone (Difco)	5 g
Yeast extract (Difco)	2.5 g
NaCl	5 g
Agar	15 g

Dissolve ingredients in distilled water and dilute up to 1 liter. Adjust pH to 7.0. Inoculate the YNA medium with a single, central streak as described above. At daily intervals, examine the edge of the streak under low power (100×) of the microscope for the characteristic skeinlike outgrowths of *K. zopfii* grown on agar. Obtain pure cultures by picking carefully from the edge of the outgrowths and plating on YNA as before.

A partial enrichment of *K. gibsonii* may be achieved by preparing a preliminary enrichment in YNB incubated for 24 h at 45°C; a second subculture at 45°C is then made before inoculating and incubating YNG and YNA plates as described above. *Bacillus* spp. do not usually interfere in this modification of the method.

Isolation by Direct Plating

If they form a sufficiently high proportion of the population, *Kurthia* spp. may be isolated by direct plating on YNA (or similar media) of materials such as meat and meat products (Gardner, 1969). Surface colonies of *Kurthia* spp. are recognized by their rhizoid form (Fig. 2a) and by the typical "medusa-head" appearance of young colonies when examined at low magnification. Such colonies have a skein-like structure that is resolved into whorls with whiplike outgrowths at the edge.

It should be noted that when pure cultures are streaked on YNA, a proportion of the colonies that develop may have a granular appearance instead of the typical rhizoid form (Keddie, 1949). Therefore, it is possible that when isolating *Kurthia* from natural materials, some may be missed because they produce nonrhizoid colonies. Isolates are then examined for the characteristic properties of *Kurthia*; useful screening tests are colony form, morphology and Gram reaction, production of "bird's feather" growth on nutrient gelatin slants (Fig. 2b), and aerobic growth in glucose nutrient agar shake cultures.

Preservation of Cultures

Cultures on YNA or nutrient agar slants should remain viable for at least 6 months when stored at room temperature (about 20°C), provided that they are not allowed to dry out. Longer-term preservation (over 10 years) may be achieved by freezing on glass beads at –60 to –70°C (Jones et al., 1984). Cultures can also be preserved by freeze drying (lyophilization).

Identification

Identification as a *Kurthia* sp. may be made on the basis of the following features. Gram-
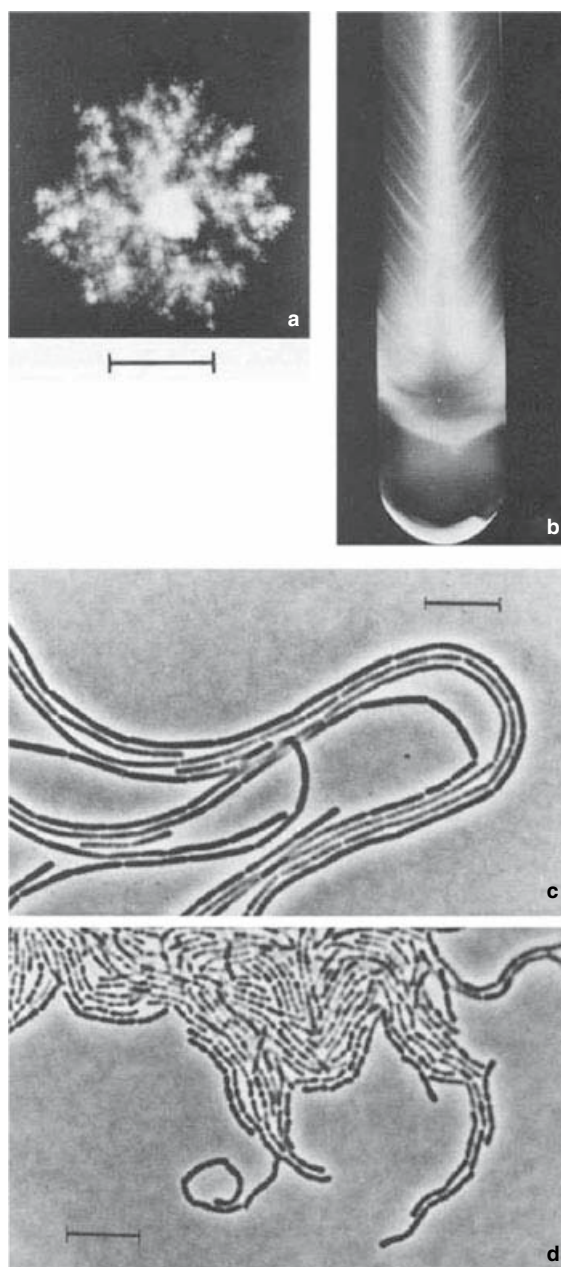
Fig. 2. (a–b) Kurthia zopfii (NCBI {9878}). (a) Rhizoid colony on yeast nutrient agar after 4 days incubation at 25°C.; Bar = 10 mm. (b) Yeast nutrient gelatin slant showing "bird's feather" type of growth; incubated 5 days at 20°C. (c–d) *Kurthia zopfii* (isolate): edge of colony on yeast nutrient agar incubated at 25°C. (c) After 24 h, showing long filaments composed of rods. (d) After 3 days, showing development of coccoid forms. Bar = 10 μm.

positive, regular, unbranched rods with rounded ends, about 0.8×3–$8 \ \mu m$ or longer, in long chains in exponential phase cultures (Fig. 2c) giving rise to coccoid cells, formed by fragmentation of the rods (Fig. 2d) or in some strains giving rise to short rods in stationary-phase cul-

tures. Strains which have been maintained in artificial culture for some time often give rise to short rods in stationary phase cultures (Shaw and Keddie, 1983a). The rods are usually motile by numerous peritrichous flagella, but nonmotile strains are known; they do not form endospores. Surface colonies on yeast nutrient agar are usually rhizoid (but granular colonial variants occur; Fig. 2a) and have a "medusa-head" appearance under low magnification (100×). They are obligate aerobes and do not form acid from glucose or other carbohydrates in peptone media. In gelatin slant cultures (see medium YNG above) inoculated with a single central streak, the growth resembles a bird's feather (Fig. 2b); the gelatin is not liquefied. They are catalase positive and grow best in the range of 25–30°C (*K. zopfii* and *K. gibsonii*) or 20–25°C (*K. sibirica*) at neutral pH.

Kurthia spp. give negative responses in most of the usual biochemical tests, e.g., indole, nitrate reduction; urease, lecithinase production; and hydrolysis of starch and esculin; however, some strains produce H_2S weakly (Jones, 1975; Shaw and Keddie, 1983a). The peptidoglycan type is A4α (L-lysine-D-aspartic acid; Belikova et al., 1980; Shaw and Keddie, 1984), abbreviated A11.31 (Schleifer and Kandler, 1972). The major isoprenoid quinones in *K. zopfii* and *K. gibsonii* are unsaturated menaquinones with seven isoprene units (MK-7; Collins et al., 1979). The peptidoglycan composition and the major fatty acids in all three species are based upon the L-Lys-D-Asp type and straight-chain saturated, *anteiso-* and *iso*-methyl-branched chain acids, respectively: the major fatty acid is 12-methyltetradecanoic (*anteiso*-$C_{15:0}$) acid in *K. zopfii* and *K. gibsonii* (Goodfellow et al., 1980), but with 13-methyltetradecanoic (*iso*-$C_{15:0}$) acid in addition in *K. sibirica* (Belikova et al., 1986). The polar lipids in *K. zopfii* and *K. gibsonii* are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine (Goodfellow et al., 1980). The G+C content is remarkably constant among isolates of all three species and lies in the range of about 36–38 mol% (Belikova et al., 1980; Belikova et al., 1986; Shaw and Keddie, 1984). Glucose is not used as a carbon + energy source, but acetate appears to be utilized by most strains of all three species (Shaw and Keddie, 1983a; Belikova et al., 1986). When supplied with a suitable source of amino acids, all species require B vitamins (Shaw and Keddie, 1983b; Belikova et al., 1986). For other characters of *K. zopfii* and *K. gibsonii*, see Table 1.

Bacteriophages isolated from *K. zopfii* are morphologically identical with the short-tailed, elongated head phages of the GA-1 species of *Bacillus* phages (Rocourt et al., 1983). The activity of these phages on *K. gibsonii* and *K. sibirica* is not known.

There is, to our knowledge, no published information on the presence of plasmid DNA in *Kurthia* spp., but a recent study demonstrated plasmid DNA in the type strain *K. zopfii* (NCTC {10597}). Further, the range of plasmids of this strain was identical to that of two isolates from sausages identified as *Brochothrix thermosphacta* (C. E. R. Dodd, personal communication). Although this observation suggests plasmid exchange between the genera, confirmation of such a phenomenon would require the demonstration of molecular identity between the plasmid complements.

Differentiation of *Kurthia* spp.

DNA-DNA hybridization studies of a few strains (some atypical) support the conclusion reached in the numerical taxonomic study of Shaw and Keddie (1983a) that *K. zopfii* and *K. gibsonii* are distinct species. Cherevach et al. (1983) showed that the yellow strains now included in the species *K. sibirica* are genomically distinct from both *K. zopfii* and *K. gibsonii*, as DNA-DNA reassociation values were low (20% with *K. zopfii* and 40% with *K. gibsonii*). These results confirm the moderate 16S rRNA gene sequence similarity among the type strains of the three species (95.8–97.6%; see phylogenetic position). The three species may be distinguished by using the characters listed in Table 2. It should be noted, however, that Shaw and Keddie (Shaw and Keddie, 1983a; Shaw and Keddie, 1983b; Shaw and Keddie, 1984) described a number of strains that had most or all of the characters of the genus *Kurthia* as defined by Keddie and Rogosa (1974), but could not be assigned to a species.

Those taxa most likely to be confused with *Kurthia* are the catalase-positive, nonsporulating Gram-positive *Brochothrix thermosphacta*, *Listeria* spp. and certain *Bacillus* spp. The organisms are easily distinguishable by a range of phenotypic properties (Table 3). *Brochothrix thermosphacta* (Sneath and Jones, 1976) is very similar to *Kurthia* in morphological features (see Davidson et al., 1968) but is nonmotile, facultatively anaerobic, and produces acid by fermentation from glucose and various other carbohydrates.

Confusion with some aerobic, saprophytic coryneform bacteria is possible but is much less likely. On nutrient agar certain chain-forming *Bacillus* spp. may give colonies somewhat similar to *K. zopfii* and have a similar morphology in exponential-phase cultures. They may also give outgrowths in nutrient gelatin, although such growth is usually, if not always, followed by liquefaction. They may be distinguished by endospore formation (detected by a test of heat resistance following growth on nutrient agar supplemented with Mn^{+2} [2 μg/ml] for at least 7

Table 1. Other features of *K. zopfii* and *K. gibsonii*.[a]

Characteristics	*K. zopfii*	*K. gibsonii*	Characteristics	*K. zopfii*	*K. gibsonii*
Acid from			Carbon + energy source, cont'd:		
Butan-1-ol	d	+	Citrate	–	d
Pentan-1-ol	d	d	Pyruvate	+	d
Hydrolysis of:			L-Citrulline	d	d
Tween 40	d	d	Oxalacetate	d	+
Tween 80	d	–	2-Oxoglutarate	d	–
Tributyrin	d	d	Ethanediol	d	d
Production of			Propanediol	–	d
H$_2$S	d	–	Adonitol	d	d
Growth at			Propan-1-ol	d	d
40°C	–	+	Butan-1-ol	+	d
Carbon + energy source			Acetaldehyde	d	d
D-Ribose	d	d	Benzaldehyde	d	d
D-Fructose	–	d	Cinnamate	d	d
N-Acetylglucosamine	+	d	Phenylacetate	d	d
Formate	–	d	Glycine	+	d
Propionate	–	d	L-Alanine	+	+
n-Hexanoate	d	d	L-Isoleucine	–	d
n-Heptanoate	d	d	L-Threonine	+	d
n-Octanoate	d	+	L-Arginine	+	d
Crotonate	d	+	L-Histidine	d	d
DL-Lactate	d	+	L-Tryptophan	–	d
DL-Glycerate	d	d	L-Hydroxyproline	d	d
Hippurate	d	–	Allantoin	d	–

Symbols and abbreviations: +, positive; –, negative; and d, 11–89% of strains are positive.

[a]The description of *K. sibirica* (Belikova et al., 1986) lacks indication of these reactions.

[b]The following reactions are identical for strains of *K. zopfii* and *K. gibsonii*:

Positive—acid from propan-1-ol, ethanediol, hydrolysis of Tween 20 and Tween 60, hippurate, uric acid; use as carbon and energy source: uridine, acetate, *n*-butyrate, succinate, fumarate, L-malate, glycerol, ethanol, L-serine, L-aspartate, L-glutamate, L-asparagine and L-proline.

Negative—acid from methanol, D-mannitol, D-sorbitol, *m*-inositol, propanediol, 2,3-butanediol, *m*-erythritol; hydrolysis of esculin, arginine, starch, tyrosine, xanthine, cellulose, casein, chitin; production of acetoin, dihydroxyacetone, sulfatase, urease, acetamidase, lecithinase, gluconate oxidation, indole; growth at 50°C; utilization of D-xylose, D-glucose, D-mannose, lactose, D-raffinose, glycogen, D-glucuronate, *n*-pentanoate, isobutyrate, tricarballylate, aconitate, DL-2-hydroxybutyrate, glyoxylate, *m*-hydroxybenzoate, uric acid, L-leucine, L-lysine, L-phenylalanine, L-tyrosine, DL-homoserine, L-cysteine, L-methionine, ethanolamine, acetamide, betaine, creatinine, L-ascorbate, *m*-erythritol, methanol, crotonol, cytosine, and xanthine.

Table 2. Differential characteristics of species of the genus *Kurthia*.

Characteristics	*K. zopfii*	*K. gibsonii*	*K. sibirica*
Growth at 45°C	–	+	–
Survival at 55°C for 20min	–	+	ND
Acid from ethanol	+	–	–
Acid from glycerol	–	+	W
Acid from fructose	–	W	+
Acid from ribose	w	W	–
Colonies, yellow or cream	–	+	+
Deoxyribonuclease	–	+	ND
Ribonuclease	+	–	ND
Phosphatase	–	+	+
4-Amino-*n*-butyrate used as carbon and energy source	–	+	ND
Pantothenic acid required	+	–	+
Nicotinic acid and pyridoxal-5-phosphate required	–	–	+

Symbols and abbreviations: +, positive; –, negative; w, weak reaction; and ND, not determined.

days). Those species most readily confused with *Kurthia*, e.g., *Bacillus cereus* subsp. *mycoides*, are facultatively anaerobic, produce acid from glucose, and liquefy gelatin. Many aerobic coryneform bacteria (e.g., *Arthrobacter* spp.), like fresh *Kurthia* isolates, give coccoid cells in stationary-phase cultures, but the appearance in exponential phase cultures is quite distinct. In

Table 3. Key phenotypic features differentiating *Kurthia* sp. and phenotypically similar bacteria associated with meat and meat products.

Characteristics	*Kurthia*	*Listeria*	*Brochothrix*	*Bacillus*
Strictly aerobic	+	−	−	d
Facultative/microaerophilic	−	+	+	+*
Lactate as fermentation product of glucose	−	+	+	d
Endospores	−	−	−	+
Diamino acid in peptidoglycan	Lys	*meso*-A2pm	*meso*-A2pm	mainly *meso*-A2pm

Symbols and abbreviations: +, positive; −, negative; Lys, lysine; d, 11–89% of strains are positive; *, exceptions are known; and A2pm, diaminopimelic acid.
From Holzapfel (1992).

coryneform bacteria, the rods are irregular in form, may show rudimentary branching, and commonly occur in V formations but never in chains.

The cellular fatty acid composition has been used as an aid to the identification of some asporogenous, aerobic Gram-positive rods (Bernard et al., 1991). Still treated as members of the coryneform group of organisms, the two strains of *Kurthia* could be distinguished from strains of *Listeria*, *Rothia*, *Jonesia*, *Oerskovia* and *Propionibacterium* by the predominance of *i*-$C_{15:0}$ over the second predominant fatty acid *ai*-$C_{15:0}$.

A molecular approach has been used to discriminate members of *Listeria* from *Kurthia zopfii* ATCC 33403 (Edmond et al., 1993). A cloned rrn operon probe, consisting of the 3′ terminus of the 16S rRNA gene, the spacer element and the 5′ terminus of the 23S rRNA gene of *Listeria monocytogenes* was found to be genus-specific under stringent conditions in dot blot analyses as well in a heteroduplex nucleic acid enzyme-linked immunoabsorbent assay.

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CHAPTER 1.2.16

The Genus *Bacillus*—Nonmedical

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History

One of the earliest bacteria to be described was "*Vibrio subtilis*" by Ehrenberg in 1835. In 1872, Cohn renamed the organism *Bacillus subtilis* (Gordon, 1981). That organism was a charter member of a large and diverse genus, initiated by Cohn, that is part of the family Bacillaceae. This family's distinguishing feature is production of endospores, which are round, oval, or cylindrical highly refractile structures formed within bacterial cells. Spores were first described by Cohn in *subtilis* and later by Koch in the pathogen, *B. anthracis* (the only major pathogen of vertebrates in the genus). Cohn demonstrated the heat resistance of spores of *B. subtilis* and Koch first described in *B. anthracis* the developmental cycle of sporeformers, vegetative cell to spore and spore to vegetative cell (Keynan and Sandler, 1983). For the reasons of unusual spore resistance to chemical and physical agents; the developmental cycle; ubiquity of its members; and *B. anthracis* pathogenicity, the genus *Bacillus* attracted early interest which has continued since.

The endospore, either as the free spore or as the structure within the vegetative cell, in which case the whole entity is referred to as a sporangium, is readily detected using the phase contrast microscope (see Fig. 1). This is because the spore at a point in the life cycle (to be detailed later) becomes highly refractile. Early workers used stains and special conditions (such as prolonged heating) to colorize the chemically impermeable spore (Doetsch, 1981). However, a Gram-stain is sufficient to determine the presence of spores because the spore remains unstainable while the vegetative cells or the vegetative part of the sporangia will stain. Because of this ease of microscopic detection of the spore and its heat resistance, many different endosporeformers can be easily found. Using any habitat—soil, water, food, etc.—as the source, sporeformers can be readily isolated by suspending a sample in water and heating at 80°C for 10 to 30 min. Vegetative cells and other resting forms such as cysts and exospores are usually killed at that temperature. The heat-resistant endospore can then be plated on appropiate media and isolates recovered in 24 to 48 h. An idea of the kinds of habitats from which *Bacillus* species have been isolated can be obtained from Table 1. Heating the inoculum, when used in conjunction with cultivation at different temperatures, hydrogen ion concentrations, degrees of aeration, and substrates, has resulted in isolating many different species of endosporeformers. The media used for the isolation and cultivation of *Bacillus* species are listed in Table 2.

More often than not since the discovery of bacteria (and in every case since 1913), the possession of an endospore has been used as a premier characteristic in keys for the classification of bacteria. The family Bacillaceae was first formulated by Fisher in 1895 (Gordon, 1981). The features of the members of the genus *Bacillus* that distinguish it from other Bacillaceae (all endosporeformers) are their aerobic nature, which may be strict or facultative, rod shape, and catalase production. The other genera of sporeformers include *Sporolactobacillus*, which is microaerophilic and catalase-negative; *Clostridium*, anaerobic but does not reduce sulfate; *Desulfotomaculum*, anaerobic but does reduce sulfate; *Sporosarcina*, a coccus; and *Thermoactinomycetes*, which while forming endospores displays typical actinomycete characteristics.

General Taxonomic Considerations

Like the sirens of Greek mythology enticing the unsuspecting sailors, *Bacillus* species have captured the curiosity of many microbiologists. The first 107 years of the efforts to classify and identify members of the genus *Bacillus* is chronicled by R.E. Gordon (1981) who with her colleagues (Gordon et al., 1973; Smith et al., 1946, 1952) made many significant contributions on which the current classification (Claus and Berkeley, 1986) was built. The early attempts were "on

This chapter was taken unchanged from the second edition.

Fig. 1. *B. megaterium* sporeforming cells as seen in the phase contrast (A) or in the interference contrast (B) microscope. The refractile bodies in the center are the spores. Bar = 5 μm.
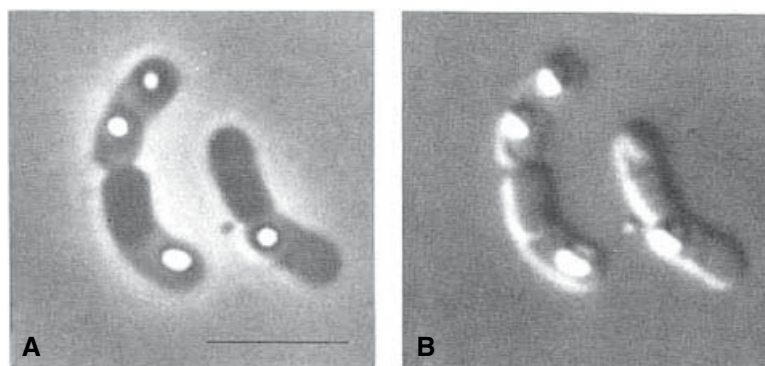
Table 1. Origins of isolates of *Bacillus* species.

Name of *Bacillus* species	Habitats from which isolated
B. subtilis	Soil, water
B. acidocaldarius	Thermal acid water and soil
B. alcalophilus	pH 10 enrichment from soil
B. alvei	Soil, diseased bee larvae
B. amylolyticus	Soil
B. anthracis	Anthrax-diseased animals
B. azotoformans	Soil
B. badius	Feces, foods, marine sources
B. brevis	Soil, foods
B. cereus	Soil, foods
B. circulans	Soil
B. coagulans	Acid foods
B. fastidiosus	Soil, poultry litter
B. firmus	Soil, salt marshes
B. globisporus	Soil, water
B. insolitus	Soil
B. larvae	Diseased bee larvae
B. laterosporus	Soil, water
B. lautus	Soil, feces
B. lentimorbus	Diseased honeybee larvae
B. lentus	Soil, foods
B. licheniformis	Soil
B. macerans	Plant materials, food
B. macquariensis	Subantarctic soil
B. marinus	Marine sediment
B. megaterium	Soil
B. mycoides	Soil
B. pabuli	Soil, fodder
B. pantothenicus	Soil
B. pasteurii	Soil, water, sewage
B. popilliae	Diseased scarabid beetles
B. psychrophilus	Soil, water
B. pumilus	Soil
B. schlegelii	Lake sediment
B. sphaericus	Soil, water sediments, foods
B. stearothermophilus	Soil, hot spring, foods
B. thermoglucosidasius	Soil
B. thuringiensis	Soil, foods
B. validus	Soil

Based on Claus and Berkeley (1986).

rocky shoals" because a classification based on only the two characteristics of aerobic growth and endospore formation resulted in grouping together many bacteria possessing different kinds of physiology and occupying a variety of habitats. This heterogeneity in physiology, ecology, and genetics makes it difficult to categorize the genus or to make generalizations about it. The range of physiological life styles is impressive: degraders of most all substrates derived from plant and animal sources including cellulose, starch, proteins, agar, hydrocarbons, and others; antibiotic producers; heterotrophic nitrifiers; denitrifiers; nitrogen fixers; iron precipitators; selenium oxidizers; oxidizers and reducers of manganese; facultative chemolithotrophs; acidophiles; alkalophiles; psychrophiles, thermophiles and others (Slepecky, 1972; Norris et al., 1981; Claus and Berkeley, 1986) (see Table 2). Because of this vast diversity of physiological types, our knowledge of sporeformer ecology is slight (Slepecky, 1972; Norris et al., 1981; Slepecky and Leadbetter, 1977, 1984). In the main, sporeformers as part of the zymogenous flora of the soil are viewed as opportunists. Upon access to the proper germinants and substrates for subsequent outgrowth, they will actively contribute to and participate in the various microhabitats which make up the soil's heterogeneous environment. Aerial distribution of the dormant spores may explain the occurrence of *Bacillus* species in most habitats examined. This diversity was apparent even with classical phenotypic characterizations based primarily on morphology (particularly size and position of the endospore within the vegetative cell), nutrition; growth characteristics; and various substrate utilization and physiological assessments.

At one time, 145 species made up the genus (Gordon, 1981). The understanding of the genus has been improved by augmenting the phenotypic characterizations with measurements of the DNA base composition and DNA-DNA hybridigation. Currently, there are listed in *Bergey's*

Table 2. Media used for the isolation and cultivation of *Bacillus* species.

B. acidocaldarius	Part A: $(NH_4)_2SO_4$, 0.4; $MgSO_4$, 1.0; $CaCl_2 \cdot 2H_2O$, 0.5; KH_2PO_4, 6.0; distilled H_2O, 1 liter; pH adj. to 4.0 Part B: glucose, 2.0; yeast ext., 2.0 distilled H_2O, 1 liter Combine A and B after sterilization
B. alcalophilus	Part A: glucose, 1.0; peptone, 5.0; yeast ext., 5.0; KH_2PO_4, 10.0 $MgSO_4 \cdot 7H_2O$, 0.2, distilled H_2O, 900ml. Part B: $Na_2CO_3 \cdot 10H_2O$, 20; distilled H_2O, 100ml. Combine A and B after sterilization (final pH = 10.5)
B. azotoformans	Peptone, 10.0; $Na_2HPO_4 \cdot 12H_2O$, 3.6; $MgSO_4 \cdot 7H_2O$, 0.03; $MnSO_4 \cdot H_2O$, 0.05; KH_2PO_4, 1.0; NH_4Cl, 0.5; $CaCl_2 \cdot 2H_2O$, 0.1; distilled H_2O, 1 liter.
B. brevis	K_2HPO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.02; NaCl, 0.02; $FeSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot H_2O$, 0.01; betaine, betaine·HCl or valine, 0.05M; agar, 16.0; distilled H_2O, 1 liter.
B. fastidiosus	K_2HPO_4, 0.8; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.05; $CaCl_2 \cdot 2H_2O$, 0.05; $FeSO_4 \cdot 7H_2O$, 0.015; $MnSO_4 \cdot H_2O$, 0.01; uric acid, 10.0; distilled H_2O, 1 liter.
B. lentus	Peptone, 10.0; meat ext., 10.0; agar, 15.0; distilled H_2O, 1 liter. adj. pH to 7.0–7.5; after sterilization, add 100g urea, steam for 10min.
B. licheniformis	Peptone, 5.0; meat ext., 3.0; KNO_3, 80.0; distilled H_2O, 1 liter; adj. pH to 7.0; fill glass-stoppered bottle to top for anaerobic conditions.
B. marinus	Peptone, 5.0; yeast ext., 1.0; $FePO_4 \cdot 4H_2O$, 0.01; agar, 15.0; aged sea water, 750ml; distilled H_2O, 250ml; adj. pH to 7.6.
B. pantothenicus	Nutrient broth + 4% (w/v) NaCl
B. pasteurii	Nutrient broth + 2% (w/v) urea
B. schlegelii	$Na_2HPO_4 \cdot 2H_2O$, 4.5; KH_2PO_4, 1.5; NH_4Cl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.01; ferric ammonium citrate, 0.005; $NaHCO_3$, 0.5; trace element soln, 5ml ($ZnSO_4 \cdot 7H_2O$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.03; H_3BO_3, 0.3; $CoCl_2 \cdot 6H_2O$, 0.02; $CuCl_2 \cdot 2H_2O$, 0.001; $NiCl_2 \cdot 6H_2O$, 0.02; $Na_2MoO_4 \cdot 2H_2O$, 0.03; distilled H_2O, 1 liter) Other: 65C, atmosphere of 0.05atm. O_2 + 0.01atm. CO_2 + 0.45atm. H_2
B. stearothermophilus	Nutrient agar; incubate cultures at 55°C

[a]Numerical amounts are grams unless specified.
[b]Most other *Bacillus* cultures will grow on nutrient broth and nutrient agar.
Based on Claus and Berkeley (1986).

Table 3. Major distinguishing characteristics of some *Bacillus* species.

Thermophile	*B. stearothermophilus*	Hydroxy aromatic compounds degrader	*B. gordonae*
	B. thermodenitrificans		
	B. caldotenax	Nitrate reduction to N_2	*B. azotoformans*
Thermophilic acidophiles	*B. coagulans*		*B. licheniformis*
	B. acidocaldarius	Growth restricted to uric acid, allantoin or allantoic acid	*B. fastidiosus*
Psychrophiles	*B. psychrophilus*	Growth at high pH and NH_4Cl	*B. pasteurii*
	B. macquariensis	Requires pantothenic acid	*B. pantothenicus*
	B. globisporus	Requires biotin	*B. pumilus*
	B. insolitus	Produces heat stable glucosidase	*B. thermoglucosidasius*
	B. psychrosaccharolyticus	Insect pathogens[a]	*B. thuringiensis*
Alkalophile	*B. alcalophilus*		*B. larvae*
Facultative chemolithotroph	*B. schlegelii*		*B. popilliae*
Nitrogen fixers	*B. polymyxa*		*B. sphaericus*
	B. macerans		*B. lentimorbus*
	B. azotofixans	Human and animal pathogen	*B. anthracis*
Alginate degraders	*B. alginolyticus*		*B. cereus*
	B. chrondrotinus		
Aromatic acid and phenol degrader	*B. benzoevorans*		

[a]See Chapter 77.

Table 4. DNA-base composition and sources of the type strains of *Bacillus* species.

Bacillus species	GC content (mol%)		Culture collection number				
	Tm[a]	BD[b]	ATCC[d]	DSM	NCIB	NCTC	NRRL
acidocaldarius	60.3	62.3	27009	446	11725		NRS1607
alcalophilus	37.0	36.7	27647	485	10436	4553	B14309
alvei	44.6	46.2	6344	29	9371	6352	B383
amylolyticus	ND[c]	53.0		3034			NRS290
anthracis	33.2	ND	14578		9388	10340	
azotoformans	ND	39.0	29788	1046			B14310
badius	43.8	43.5	14574	123	9364	10333	NRS663
brevis	47.3	47.4	8246	30	9372	2611	NRS604
cereus	35.7	36.2	14579	31	9373	2599	B3711
circulans	35.5	35.4	4513	11	9374	2610	B380
coagulans	47.1	44.5	7050	1	9365	10334	NRS609
fastidiosus	35.1	35.1	29604	91	11326		
firmus	41.4	40.7	14575	12	9366	10335	NRS613
globisporus	39.8	39.7	23301	4	11434		NRS1533
insolitus	35.9	36.1	23299	5	11433		
larvae	ND	50.0	9545				B2605
laterosporus	40.2	40.5	64	25	9367	6357	NRS314
lautus	ND	50–52		3035			NRS666
lentimorbus	37.7	ND	14707	2049	11202		B2522
lentus	36.3	36.4	10840	9	8773	4824	B396
licheniformis	46.4	44.7	14580	13	9375	10341	NRS1264
macerans	52.2	53.2	8244	24	9368	6355	B172
macquariensis	39.3	41.6	23464	2	9934	10419	B14306
marinus	37.6	38.0	29841	1297			B14321
megaterium	37.3	37.6	14581	32	9376	10342	B14308
mycoides	34.2	34.1	6462	2048			NRS273
pabuli	ND	48–50		3036			NRS924
pantothenicus	36.9	36.8	14576	26	8775	8162	NRS1321
pasteurii	38.5	38.4	11859	33	8841	4822	NRS673
polymyxa	44.3	45.6	842	36	8158	10383	NRS1105
popilliae	41.3	ND	14706	2047			B2309
psychrophilus	39.7	40.5	23304	3			NRS1530
pumilus	41.9	40.7	7061	27	9369	10337	NRS272
schlegelii	64.6	66.3	43741	2000			
sphaericus	37.3	37.1	14577	28	9370	10338	
stearothermophilus	51.9	51.5	12980	22	8923	10339	B1172
subtilis	42.9	43.1	6051	10	3610	3610	NRS744
thermoglucosidasius	45–46	ND	43742	2542			B14516
thuringiensis	33.8	34.3	10792	2046	9134		NRS996
validus	ND	53–54		3037			NRS1000

[a]Tm, GC content by thermal melting.
[b]BD, GC content by buoyant density.
[c]ND, not determined.
[d]ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; NCIB, National Collection of Industrial Bacteria; NCTC, National Collection of Type Cultures; NRRL, Northern Regional Research Laboratory.

Manual of Systematic Bacteriology (BMSB) 40 recognized species (Claus and Berkeley, 1986), Table 4 lists these with their GC content. There are several validly published new species shown to be genetically and phenotypically distinct from other *Bacillus* species that have not been described in *Bergey's Manual*. These include *B. pulvifaciens* (Nakamura, 1984); *B. alginolyticus* and *B. chrondrotinus*, two alginate-degrading species, (Nakamura, 1987); *B. smithii* (Nakamura et al., 1988); *B. thermoleovorans*. an obligately thermophilic hydrocarbon-utilizing organism (Zarilla and Perry, 1987); *B. benzoevorans*, an aromatic acid and phenol degrader (Pichinoty et al., 1984); and *B. gordonae*, degrader of hydroxy aromatic compounds (Pichinoty et al., 1986).

There are more than 200 species of *Bacillus* in the category "*Species Incertae Sedis*" (Claus and Berkeley, 1986). These have been inadequately described or the orginal isolates have been lost. Presumably, these can be revived for listing in *Bergey's Manual* after reisolation and more detailed studies. For example, after extensive reconsideration of phenetic and molecular data it has been proposed that *B. flexus*, *B. fusiformis*, *B. kaustophilus*, *B. psychrosaccharolyticus*, *B.*

simplex (Priest et al., 1988), and *B. thiaminolyti-cus* (Nakumura, 1989) be recognized.

The literature contains many important experiments done with *Bacillus* isolates that have not yet been properly identified to species such as: *Bacillus* sp. strain SGI, a manganese-oxidizing and reducing organism (Johannes et al., 1986; Rosson and Nealson, 1982); *Bacillus* sp. strain C-59 and *Bacillus* sp. strain N-6, both alkalophilic organisms with unusual bioenergetic properties (Kitada and Horikoshi, 1987; Kitada et al., 1989); *Bacillus* sp. strain Gx6638, a novel alkaline and heat stable serine protease-secreting strain (Durham et al., 1987); and *Bacillus* sp. strain MGA3, a thermophilic methanol-utilizing species, mutants of which are capable of producing large amounts of lysine (Guettler and Hanson, 1988; Schendel et al., 1989).

An extensive list of phenetic characters of most members of the genus have been complied and procedures for the isolation and identification of individual species have been presented (Gordon et al., 1973; Berkeley and Goodfellow, 1981; Norris et al., 1981; Claus and Berkeley, 1986). Summaries of one such rendition are shown in Tables 5 and 6 (Norris et al., 1981).

The GC content (32–69 mol%) of the known *Bacillus* species as well as DNA hybridization experiments have revealed the heterogeneity of the genus (Priest, 1981; Fahmy et al., 1985) (see Table 4). Not only is there variation from species to species but there are differences in GC content within strains of a species identified on other bases. For example, the GC content of the *B. megaterium* group ranges from 36 to 45% (Hunger and Claus, 1981). It is thus understandable

Table 5. Simplified key for the tentative identification of typical strains of *Bacillus* species.

1. Catalase: positive 2
              negative 17
2. Voges-Proskauer: positive 3
              negative 10
3. Growth in anaerobic agar: positive 4
              negative 9
4. Growth at 50°C: positive 5
              negative 6
5. Growth in 7% NaCl: positive .... *B. licheniformis*
              negative .... *B. coagulans*
6. Acid and gas from glucose (inorganic N): positive.......... *B. polymyxa*
              negative......... 7
7. Reduction of NO₃ to NO₂: positive........................... 8
              negative .... *B. alvei*
8. Parasporal body in sporangium: positive .... *B. thuringiensis*
              negative .... *B. cereus*
9. Hydrolysis of starch: positive .... *B. subtilis*
              negative .... *B. pumilus*
10. Growth at 65°C: positive .... *B. stearothermophilus*
              negative 11
11. Hydrolysis of starch: positive 12
              negative 15
12. Acid and gas from glucose (inorganic N): positive............... *B. macerans*
              negative 13
13. Width of rod 1.0μm or greater: positive .... *B. megaterium*
              negative.................. 14
14. pH in V-P broth <6.0: positive .... *B. circulans*
              negative .... *B. firmus*
15. Growth in anaerobic agar: positive....................... *B. laterosporus*
              negative 16
16. Acid from glucose (inorganic N): positive .... *B. brevis*
              negative .... *B. sphaericus*
17. Growth at 65°C: positive .... *B. stearothermophilus*
              negative 18
18. Decomposition of casein: positive............................. *B. larvae*
              negative............................. 19
19. Parasporal body in sporangium: positive .... *B. popilliae*
              negative .... *B. lentimorbus*

[a]Numbers on the right indicate the number (on the left) of the next test to be applied until the right-hand number is replaced by a species name.
From Norris et al. (1981).

Table 6. Summary of the characters used in the simplified key for *Bacillus* species.

	Catalase	V-P reaction	Growth in anaerobic agar	Growth at 50°C	Growth in 7% NaCl	Acid and gas in glucose	NO_3 reduced to NO_2	Starch hydrolyzed	Growth at 65°C	Rods 1.0μm wide or wider	pH in V-P medium <6.0	Acid from glucose	Hydrolysis of casein	Parasporal bodies
B. megaterium	+	−	−	−	+	−	V	+	−	+	V	+	+	−
B. cereus	+	+	+	−	+	−	+	+	−	+	+	+	+	V
B. thuringiensis	+	+	+	−	+	−	+	+	−	+	+	+	+	+
B. licheniformis	+	+	+	+	+	−	+	+	−	−	V	+	+	−
B. subtilis	+	+	−	+	+	−	+	+	−	−	V	+	+	−
B. pumilus	+	+	−	+	+	−	−	−	−	−	+	+	+	−
B. firmus	+	−	−	−	+	−	+	+	−	−	−	+	+	−
B. coagulans	+	+	+	+	−	−	V	+	−	V	+	+	V	−
B. polymyxa	+	+	+	−	−	+	+	+	−	−	V	+	+	−
B. macerans	+	−	+	+	−	+	+	+	−	−	−	+	−	−
B. circulans	+	−	V	+	V	−	V	+	−	−	V	+	V	−
B. stearothermophilus	V	−	−	+	−	−	V	+	+	V	+	+	−	
B. alvei	+	+	+	−	−	−	−	+	−	V	+	+	+	−
B. laterosporus	+	−	+	+	−	−	+	−	−	−	−	+	+	+
B. brevis	+	−	−	+	−	−	V	−	−	−	−	+	+	−
B. larvae	−	−	+	−	+[a]	−	V	−	−	−	−	+	+	−
B. popilliae	−	−	+	−	+[a]	−	−	−	−	−	−	+	−	+
B. lentimorbus	−	−	+	−	−	−	−	−	−	−	−	+	−	−
B. sphaericus	+	−	−	−	V	−	−	−	−	V	−	−	V	+

+, Greater than 85% of strains examined by Gordon, Haynes, and Pang (1973) positive; −, greater than 85% of strains negative; V, variable character.

[a]Growth in 2% NaCl agar.

that Priest et al. (1981, 1988), who have conducted extensive numerical analysis of many unit characters in addition to the DNA studies, have proposed that the genus *Bacillus* be split into multiple genera, since the intrageneric heterogeneity is as great as exists in most bacterial families. Priest et al. (1988) assigned 80 organisms of species rank to five or more cluster groups. Their studies reemphasized the heterogeneity of the *B. brevis*, *B circulans*, *B. coagulans*, *B. sphaericus*, and *B. stearothermophilus* groups.

A variety of techniques have been employed to find either a simple approach to *Bacillus* taxonomy or a quick and painless identification methodology. Assessment of lipid analyses (reviewed by Minnikin and Goodfellow, 1981) indicated that *Bacillus* could not be separated into discrete groups. On the other hand, some species could be delineated from others. For example, *B. acidocaldarius* could be characterized by its menaquinone (nine isoprenoid units, MK-9), cyclohexyl fatty acids, triterpenes, and complex lipids.

Using the API System (Analytab Products Incorporated) (a rapid identification system wherein many standardized biochemical assessments can be made on test strips) and some

supplementary classical determinants, Logan and Berkeley (1981, 1984) have examined 1,075 *Bacillus* strains. They were able to show that the API System tests were more reproducible than the classical tests.

Pyrolysis gas-liquid chromatography has been applied to the problems of *Bacillus* taxonomy (O'Donnell and Norris, 1981; O'Donnell et al., 1988). Although there are still some problems with the technique, some promise for its use in classification and identification has been shown. For example, as with DNA-DNA hybridization studies, a separation has been made between *B. subtilis* and *B. amyloliquefaciens*. When gasliquid chromatography was applied to examine the subgroups of *B. megaterium*, data were obtained that confirmed the heterogeneity of the group even though there was some difficulty in resolving relationships within the group.

Shute et al. 1984 have used Curie-point pyrolysis mass spectrometry as a taxonomic tool. *B. subtilis*, *B. pumilus*, *B. licheniformis*, and *B. amyloliquefaciens* could be separated using data obtained from nonsporulating cultures (those grown on nutrient agar); however, such was not the case with cultures sporulating on nutrient agar plus manganese.

Ribosomal RNA Sequencing

The most effective approach to *Bacillus* taxonomy may be analysis of 16S rRNA molecules by oligonucleotide sequencing (Fox et al., 1977; Stackebrandt and Woese, 1979). That technique holds much promise for leading microbial taxonomy into natural phylogenetic relationships. However, traditional taxonomists may be dismayed to find that *Bacillus* species show kinship with nonsporeforming species. Early studies with this powerful tool showed a close relationship among *Bacillus*, *Planococcus*, *Sporosarcina*, *Staphylococcus*, and *Thermoactinomycetes* (Stackebrandt et al., 1987; Stackebrandt and Woese, 1981). In a recent study 16S rRNA cataloging showed that *B. subtilis* and other ellipsoidal-sporeforming species, *B. cereus*, *B. megaterium*, and *B. pumilus*, formed a coherent cluster, while the round-sporeforming species, *B. sphaericus*, *B. globisporus*, and "*B. aminovorans*" did not cluster. Furthermore, the latter group were closer phylogenetically to nonsporeforming organisms as follows: *B. sphaericus* to *Caryophanon latum*; *B. globisporus* to *Filibacter limicola*; *B. pasteuri* to *Sporosarcina urea* and "*B. aminovorans*" to *Planococcus citreus*. Cell wall composition agreed except with the last case. *B. stearothermophilus* fell outside the main *Bacillus* cluster and showed some relationship to *Thermoactinomycete vulgaris* (Stackebrandt et al., 1987).

In a more recent 16S rRNA sequencing survey, three major *Bacillus* taxonomic cluster groups were defined (Jurtshuk et al., 1989). This was accomplished by determining complete or partial sequences of 16S RNA on 35 recognized neotype reference strains or type species by the technique of Lane et al. 1985. The partial sequences analyzed typically exceeded 1,100 nucleotides. Phylogenetic analyses were performed using three different approaches (Sneath and Sokal, 1973; Fitch and Margoliash, 1967; Saitou and Nei, 1987) which showed three major groupings of *Bacillus* spp., hereinafter referred to as clusters I, II, and III (see Table 7). The 16S rRNA *Bacillus* cluster groups were quite different from those previously noted by Stackebrandt et al. (1987). This is revealed by direct comparison to the commonly used morphological groupings (see Table 7). Except for morphological group II and the Unassigned Subgroup 2E, all strains sequenced fell into the *B. subtilis* cluster I grouping. *Bacillus* strains of morphological group II fell into all three 16S rRNA cluster groups and *B. macquariensis*, unlike other psychrophiles, fell into the *B. alvei* cluster II group.

Comparative 16S rRNA analyses on thermophilic and psychrophilic *Bacillus* strains (Wisotzkey et al., 1989) showed that the thermophiles, *B. stearothermophilus*, *B. thermodenitrificans* and *B. caldotenax* formed a subgroup within the *B. subtilis* cluster but separate from both the "thermotolerant" mesophilic, *B. subtilis* and *B. licheniformis* strains, and the moderate thermophile, *B. coagulans*. The psychrophilic strains, *B. psychrophilus* and *B. insolitus*, fell into cluster I while *B. macquariensis* fell into cluster II.

Because several species were included in the current study that had previously been examined by 16S rRNA oligonucleotide cataloging, it is possible to compare the two data sets directly. As a result, it is possible to augment the membership of cluster I to include *B. fastidiosus*, *B. firmus*, *B. badius* and *B. pasteurii* (C. B. Woese, personal communication). In addition, it is extremely likely that at least two nonsporeforming strains, *Planococcus citreus* (Stackebrandt and Woese, 1979) and *Filibacter limicola* (Clausen et al., 1985), as well as *Sporosarcina ureae* (Pechman et al., 1976), are properly regarded as members of cluster I.

Table 7. *Bacillus* 16S rRNA cluster groups.

Morphological group	*B. subtilis* cluster I	*B. alvci* cluster II	*B. brevis* cluster III
I	*B. subtilis, B. cereus, B. licheniformis, B. pumilus, B. megaterium* strain Mohb, *B. coagulans, B. smithil*		
II	*B. circulans, B. larvae, B. stearothermophilus*	*B. alvei, B. polymyxa, B. macerans, B. azotofixans, B. pulvifaciens*	*B. brevis, B. laterosporus*
III	*B. sphaericus*		
Subgroup A	"*B. thiaminolyticus*," *B. alcalophilus*		
Subgroup B	*B. lentus*		
Subgroup C	*B. freundenreichii*, "*B. aneurinolyticus*"		
Subgroup D	*B. pantothenticus*		
Subgroup E1	"*B. psychrophilus*," *B. insolitus*		
Subgroup E2		*B. macquariensis*	

Table provided by Peter Jurtshuk.

Life Cycle; Sporulation and Germination as Models for Differentiation

Introduction

The processes of resting cell formation and the change back to the vegetative cell in a variety of prokaryotes (Losick and Shapiro, 1984) present excellent models for studying differentiation, with the added attendant advantages of microbial systems: ease of handling, use of large numbers of cells, fast growth, synchrony, and availability of mutants. The endospore models were recognized early and, therefore, more knowledge has been accumulated using them than with other prokaryotic systems. As the attempt to categorize the many species of *Bacillus* has had a long history, so have the efforts to unravel the many aspects of the life cycle of *Bacillus* (for a historical treatment see Keynan and Sandler, 1983). Because of the enormous literature in this area, the present treatment of the life cycle relies mainly on reviews of the subject and covers mainly highlights of germination and sporulation.

The cycle of germination, outgrowth, growth, and sporulation (shown schematically in Fig. 2) has been studied from many different aspects with many different species of spore-formers but because of the genetic versatility of *B. subtilis*, most work has focused on this species.

Germination and Outgrowth

Free spores usually must be activated for germination. Activation is a reversible process which conditions the spore for germination and increases the number of spores undergoing germination as well as the rate of germination. Spores can be activated by a variety of treatments, notably exposure to heat. During activation there is a loss of some coat protein, dipicolinic acid (DPA), and Zn^{2+} along with an increase in membrane fluidity. Germination, the breaking of the spore's highly dormant state, follows (recent reviews on germination include Setlow, 1983, and Foster and Johnstone, 1989). A series of degradative reactions is triggered in an unknown manner by simple compounds such as certain amino acids and ribosides or mixtures (no universal germinant has been described) or certain nonnutrient conditions, and can be monitored by the loss of spore refractility as seen in the phase-contrast microscope and by decrease in optical density. No metabolic activity can be detected during the first 2 min of germination of spores that require alaine or glucose for germination. Generation of ATP or production of known metabolic products of these initiators has not been found. Mutants deficient in key glycolytic pathway enzymes can germinate, thus ruling out glycolysis in the case of spores requiring glucose for germination. The same spores can be germinated by nonmetabolizable glucose analogs as well. However, metabolism may play a role in the germination of *B. fastidiosus*, whose
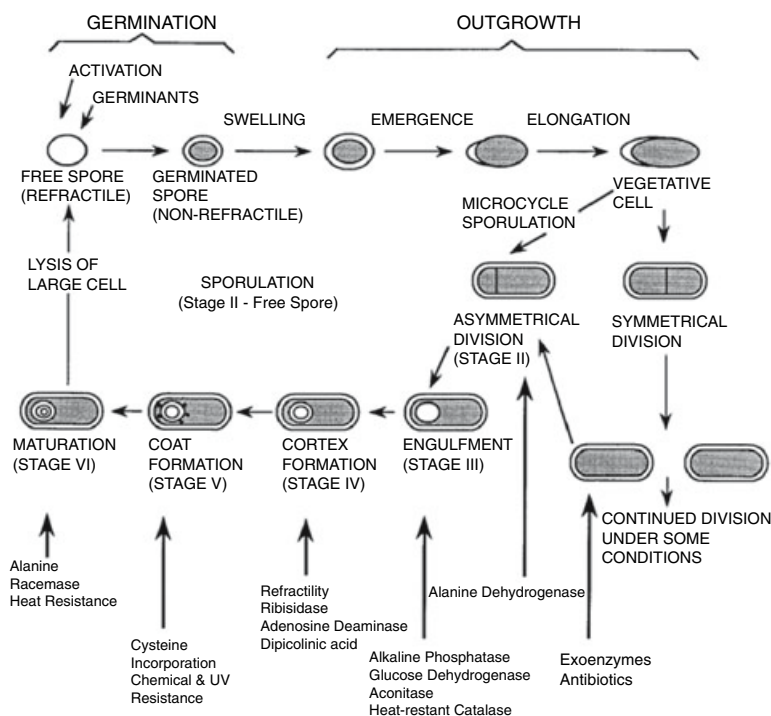
Fig. 2. Cycle of germination, outgrowth, and sporulation of a typical sporeforming bacterium. Also shown are some biochemical and physical events associated with various stages. (modification of a figure in Slepecky, R.A. (1978)).

spores can only be germinated with uric acid, which is the main carbon source for these unique organisms (Aoki and Slepecky, 1973). One hypothesis suggests that germinants act on receptor proteins, possibly in the inner membrane, which then undergo conformational changes that alter permeability (Foster and Johnstone, 1989). This leads to an autocatalytic loss of heat resistance and to changes that initiate metabolism, leading to vegetative growth. Another view, based on the observation that inhibition of the electron transport system affects germination, postulates that respiration and ATP create a proton motive force, lending to the establishment of a proton gradient. The proton motive force is used for transport of ions from core to cortex to neutralize other ions (Gould, 1983).

Upon germination, the spores not only lose their resistance to heat but also resistance to radiation and injurious chemicals; their stainability also increases. Concomitantly with "phase" darkening, the spores swell, break out of their coats, and exude up to 30% of their dry weight; about one-half of the exudate consists of a calcium chelate of the spore-specific substance, DPA, and the remainder consists of peptidoglycan fragments (from the action of cortex lytic enzymes) and amino acids. The earliest measurable events are the loss of calcium, DPA, and heat resistance. This is followed by metabolic events using high-energy compounds produced early in germination from energy reserves stored in the dormant spore.

RNA synthesis begins rapidly within 2 min of germination. The dormant spore lacks the ability to produce amino acids and amino acid biosynthesis is absent early in germination. During the first minutes of germination, 20% of the spore's protein is degraded, providing the source of amino acids for biosynthesis of new protein and small molecules (such as nucleotides) during outgrowth (reviewed by Setlow, 1988). The spore's enzymes are not degraded. Rather, a group of small acid-soluble proteins (SASP) are the source of the amino acids. These unique proteins, located in the core and sensitive to proteolysis, conprise 8 to 20% of the protein in the spore. Their molecular weight is low (5–11 kDa) and although they are not histones, they bind to the spore DNA. The proteins are degraded by a unique protease that has an absolute specificity for these proteins. They are synthesized late in sporulation. Several of the five known SASP genes (referred to as *ssp*) have been cloned and in addition to coding proteins supplying amino acids in germination their products may have other roles. One has been shown to be involved in ultraviolet (UV) resistance.

Many germination mutants (abnormal germination phenotypes) have been described (reviewed by Moir et al., 1986; Foster and Johnstone, 1989). The *ger* (germination) genes are considered a subclass of *spo* (sporulation) loci, and are made up of several classes: I, structural genes for germination mechanism components; II, regulatory genes for class I; III, post-translation processing and assembly genes; and IV, genes for synthesizing spore structure (e.g., cortex) required for germination.

Outgrowth is the period during which the spore gradually becomes a vegetative cell and initiates new macromolecular synthesis. Genes associated with this period are *out* genes. DNA is replicated relatively late in outgrowth, just before division. The vegetative cell is then capable of undergoing various morphological and biochemical changes which lead either to a series of symmetric cell divisions if sufficient nutrients are present, whereas in stressful times (particularly nutrient limitation), subsequent spore formation or the production of a spore without intermittent cell division can occur. The latter is known as microcycle sporulation (Vinter and Slepecky, 1965).

Sporulation

Electron microscopical analyses of cells during sporulation has revealed seven stages (reviewed by Fitz-James and Young, 1969). The various stages are shown in Fig. 2. Vegetative cells are considered to be Stage 0. Upon induction input prior to actual sporulation, the nuclear material is in an axially disposed filament. This is stage I. However, since such a pattern does not appear to be unique to sporulating cells, current practice refers to cells in stages prior to stage II as pre-septation cells. Segregation of the chromatin material to the poles of the cell occurs concomitantly with the invagination of the plasma membrane in an asymmetrical position on the cell which fuses to complete the spore septum. This is stage II. The mode of formation of this septum is similar to the formation of the transverse septum of symmetric vegetative cell division. Indeed, it has been proposed that sporulation because of this and some other similarities is a modified prokaryotic division (Hitchins and Slepecky, 1969) and models for that view have been presented (Freese and Heinze, 1983). In sporulation, the division of the cell is not equal and subsequent proliferation of the larger cell's plasma membrane leads to complete engulfment of the "forespore" and liberation of the immature spore, surrounded now by a double unit membrane, into the cytoplasm of the larger cell. This completes stage III. This is a key step since this double membrane now has different trans-

port properties owing to the opposing polarity of the two membranes.

During sporulation the vegetative cell is divided into two compartments each having a different fate and each displaying different patterns of gene expression (reviewed by Setlow, 1989). At this time, the cell is "committed" to complete the process of sporulation. The small cell eventually becomes the core of the spore while the large cell, the mother cell, goes on to produce the outer protective layers of the spore and then lyses to release the spore. Cortex material similar to vegetative cell peptidoglycan (but differing in the degree of cross-linking and other aspects) is laid down between the unit membranes, and its deposition corresponds in time to the accumulation in the core of DPA and calcium. Stage IV is now completed. Studies with cortex-less mutants show that the cortex is needed for refractility of the spore (when the spores become refractile, they can be seen in the phase-contrast microscope) and for accumulation of DPA. The cortex plays a fundamental role in the dehydration of the spore (reviewed by Gould, 1983).

During Stage V, protein coats are synthesized by the mother cell. There are about 10 major coat proteins in *B. subtilis* and they are encoded by *cot* genes, seven of which are known (Losick et al., 1986). The coat proteins are placed around the outside of the forespore. In some species, an additional protein layer called an exosporium is synthesized. Since the coat may play important roles in protection of the spore and its subsequent germination, it has been the subject of many investigations (reviewed by Aronson and Fitz-James, 1976; Losick et al., 1986). Electron microscopy reveals that *B. cereus* contains an outer coat showing a cross-patched pattern, an inner pitted layer, and a thin layer, the undercoat, while other species show distinct differences. *B. subtilis* possesses a very thick multilayered coat with an outer striped layer and *B. thuringiensis* has a coat deficient in the outer cross-patched layer. Differences show up as well within the major structural polypeptides and coat-associated proteases. These differences may be responsible for the variation found in germination and certain resistant properties (other than heat and UV resistance) of various species.

During vegetative growth and subsequent sporulation, a variety of proteases are produced (reviewed by Priest, 1977). There are six extracellular proteases and at least three major intracellular proteases—ISP, esterase A, and esterase B. They may be involved with turnover of intracellular proteins, the processing of protein precursors for spore coats, or inactivation of later sporulation enzymes, as well as other functions. As with other aspects of *B. subtilis* physiology,
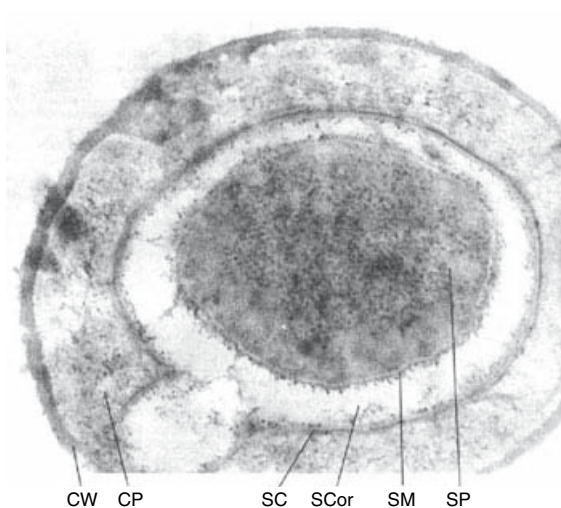
Fig. 3. Cross-section of *Bacillus megaterium* containing a spore and showing the sporangium (cell), cell protoplast (CP) and wall (CW), spore coat (SC), spore cortex (SCor), spore membrane (SM), and spore protoplast (SP). ×120,000. (Norris et al., 1981.)

there are other considerations. Even though the genes *apr*, *npr*, *epr*, and *isp*, which code for the proteases alkaline (subtilisin), neutral (metallo-) "new" serine, and major intracellular serine, respectively, can be deleted, there still is some protease activity (Sloma et al., 1988). This finding suggests that there are other unindentified proteases.

As the spore matures (stage VI), it becomes resistant to heat and to a variety of organic solvents. Final lytic enzymes lyse the sporangial or mother cell liberating the free spore (stage VII). Figure 3 shows a cross section of a mature and heat-resistant spore. If the free spore is placed among the proper nutrients it will germinate, completing the cycle. The sporulation process takes 6 to 8 h at 37°C in *B. subtilis*.

Many other biochemical and physiological events occur during sporulation in addition to those indicated in Fig. 2 and those that can be surmised to be linked with the morphologically identified stages. Some vegetative enzyme activities disappear, some remain, others are modified, and new sporulation specific enzymes are synthesized. The number of sporulation-associated events is uncertain, but the genetic evidence suggests as many as 200 genes in 40 to 50 operons are involved (Losick et al., 1986; Mandelstam and Errington, 1987; Piggot, 1989; Youngman et al., 1989). Currently the genetic map, which includes sporulation and germination genes as well as all-known vegetative cell genes, contains 700 loci, more than 300 of which have been cloned and 180 of those sequenced (reviewed by Piggott, 1989). The ordered appearance of cytological and biochemical changes

implies a sequential reading of the genome. The genetic data are consistent with there being a single linear dependent sequence to stage III with a more complex pattern of gene expression beyond that stage.

The genotype of mutants blocked at the various morphological stages in sporulation (as determined by electron microscopy) are designates *spoO*, *spoII*, *spoIII*, etc. For example, a *spoII* mutant would be arrested in stage II. Within each genotype, the designation *A*, *B*, *C*, etc. identifies different genetic locations; for example, *spoIIA*, *spoIIB* identify two separate mutations that cause arrest of developmental stage II. Eight loci have been shown to be concerned with stage 0, the five principal spoO genes being *spo0A*, *B*, *E*, *F*, and *H*. These *spo0* mutants have pleiotropic effects on the phenotype, suggesting that their gene products regulate the expression of several genes. They do not make polar septa (the asymmetric division) or Stage 0 associated products—proteases, antibiotics and transformation competent cells. There are suppressors of spo0 mutations, i.e., suppressor mutations can restore sporulation in some spo0 mutants to the wild type level. For example, *sof-I* can suppress defects in *spo0F*, *B*, and *E* (Hoch et al., 1985). Others include *arbA* and *arbB* suppressors of *spo0A* mutations; *rvtA*, a *spo0* repressor; *ssa*, a reliever of suppression of alcohols; *crs*, an alleviator of glucose repression of sporulation; and *sapA* and *sapB*. suppressors involved with alkaline phosphate induction (Losick et al., 1986). Studies on these extragenic suppressors have given insights into the role of *spo0* mutants.

Sporulation, normally repressed at high growth rates in the presence of excess nutrients, will ensue at the beginning of stationary phase (arbitrarily defined as t_0) in batch culture upon limitation of carbon, nitrogen, or phosphate (reviewed by Smith, 1989, and Sonenshein, 1989). The initiating signal for sporulation is not known. However, sporulation initiation is associated with a decrease in the intracellular GTP pool (reviewed by Freese and Heinze, 1983). Decoynine, a drug that artificially reduces GTP level, induces sporulation even in the presence of excess nutrients. There may be a connection between GTP levels and a proposed sensing mechanism as follows: *spo0A*, *B*, and *F* share significant homology and *A* and *F* products show partial homology with proteins such as OmpR, NtrC, and SfrA which are "sensing proteins" involved in the transduction of various environmental signals (Trach et al., 1985; Nixon et al., 1986). Thus, *spo0A* and *spo0F* may play a role in sensing starvation and transferring a signal to other genes (see review by Smith, 1989, on the initiation of sporulation). A gene for a protein

kinase that phosphorylates sporulation regulatory proteins Spo0A and Spo0F has been characterized (Pergo et al., 1989). The gene, *kinA*, previously known as *spo11J*, has been shown to code for a protein that is homologous to the transmitter class of proteins (Stragier, 1989). Defects in the methylation of a membrane-associated 40-kDa protein in some *B. subtilis* *spo0* mutants suggests that protein methylation also may be a part of the nutrient-sensing system (Golden and Bernlohr, 1989).

Multiple forms of RNA polymerase are of considerable importance in sporulation (reviewed by Doi and Wang, 1986; Losick et al., 1986; Losick and Kroos, 1989; Moran, 1989; Setlow, 1989; Stragier, 1989). The holoenzyme is made up of an enzyme core comprised of subunits beta, beta′ and alpha (the products of genes *rpoB*, *rpoC*, and *rpoA*, respectively) and attached sigma factors (the products of genes to be designated), regulatory proteins that determine the promoter specificity. Currently, there are nine known sigma factors, four associated with vegetative cells and five involved in sporulation (see Table 8). Losick and Pero (1981) suggested there is a succession of different sigma factors governing the transcription of the various temporal classes of sporulation genes.

The first acting sporulation sigma is ;gs;xH which is required for the initiation of sporulation and it is involved with the transcription of a later gene, *spoVG*.

A key point in sporulation is stage II, which is the result of the asymmetric division event. stage II mutants and the associated gene products or functions are as follows: *spoIIAA* σ^E processing); *IIAC* (σ^E factor); *IIE* (pro σ^E); *IIGA*σ^E processing protease); *IIGB* (p^{31}; σ^E); *IIN* (Fts homology) and *IIJ* (ntrB protein kinase) (Leighton cited in Stragier (1989), and Stragier (1989)). That the *spoIIN* product has homology with *E. coli* Fts, a protein involved in symmetrical cell division, may suggest that the level of Fts controls the asymmetric division. In turn, the functions of the other *spoII* mutations listed above implies a rather complicated mechanism for the synthesis of σ^E. The sporulation septum (stage II), dependent on *spoIIAC* (σ^F) and *spoIIE* products, is required for *spoIIGA* processing activity, i.e., conversion of pro-σ^E by proteolytic cleavage to σ^E. The important finding by Stragier (1989) of the coupling of gene expression to morphogenesis may be a recurrent theme throughout other aspects of sporulation.

After Stage II, compartmentalization of sigma factors occurs. σ^E is active both in the mother cell and forespore compartment. σ^G acts only in the forespore compartment (Setlow, 1989) while σ^K, the product of *sig K*, a composite gene from *IVCB* plus *IIC* (due to a chromosome

Table 8. *Bacillus subtilis* sigma factors.

Sigma type	Previous designation	Gene	Physiological role	Time of action	Target genes	Cognate consensus −35	Promoter sequence −10
σ^A	σ^{55}, σ^{43}	*sig A (rpoD)*	Housekeeping	$<t_0$	Many	TTGACA	TATAAT
σ^B	σ^{37}	*sig B*	Unknown *ctc* transcription	$<t_0$	Many	AGGNTT	GGNATTGNT
σ^C	σ^{32}	*sig C³*	Unknown	$<t_0$	Many	AAATC	TANTGNTNTA
σ^D	σ^{38}	*sig D*	Flagellar synthesis	$<t_0$	Flagellar genes	CTAA	CCGATAT
σ^E	σ^{34}	*sig E spoIIGB*	Engulfment; provides compartmentalization	$t_{1.5}$–$t_{3.5}$	*spoIID*	TTNAAA	CATATT
σ^F	$\sigma^{SpoIIAC}$	*sig F spoIIAC*	Asymmetric division (?)	About t_1–t_2	Unknown	Unknown but similar to σ^G	
σ^G	—	*sig G spoIIIG*	Forespore gene expression	t_3–t_5	*sspA-E-spoVA*	YGHATR	CAHWHTAH
σ^H	σ^{50}	*sig H spoOH*	Entry into stationary phase	About t_0	*spoVG*	CAGGA	GAATWWT
σ^K	σ^{27}	*spoIVCB + spoIIIC*	Mother cell gene expression	$t_{3.5}$–$t_{5.5}$	*cotA, cotD*	Unknown	

Modified from Helmann and Chamberlain (1988); Moran (1989); and Stragier (1989).

rearrangement requiring *SpoIIID* and a recombinase) acts only in the mother cell (Losick and Kroos, 1989). σ^K directs the transcription of *cotA* and *cotD*, spore coat genes.

This brief overview of some of the emerging information on regulation of gene expression during sporulation reflects the current view of a network of dependent pathways in which activation of developmental genes depends on the products of other developmental genes (Losick and Kroos, 1989).

Surface Structures of *Bacillus*

S-Layers

Crystalline surface layers of protein or glycoprotein subunits, called S-layers, are found in members of the genus *Bacillus* (reviewed by Sleytr and Messner, 1988). S-layers of individual strains of *Bacillus* have been shown to differ in molecular weight (40–200 kDa), the degree of glycosylation of the subunits, and the geometry of the S-layer lattice. For example, *B. stearothermophilus* contains one S-layer consisting of two types of glycan chains, one being a unique type of protein-carbohydrate linkage. On the other hand, *B. brevis* contains two S-layers, termed the outer wall protein (OWP) and the middle wall protein (MWP), external to the peptidoglycan layer. These form a hexagonal array in the cell wall. The nucleotide sequence of the entire MWP-OWP gene operon is known (Tsuboi et al., 1988). The gene encoding an S-layer protein of *B. sphaericus* has been cloned and sequenced (Bowditch et al., 1989). Not all *Bacillus* species contain S-layers and some strains within a species may lack such a layer. Furthermore, the type of lattice may vary from species to species and within strains of a species. *B. alvei*, *B. anthracis*, and *B. brevis* show a hexagonal array; *B. cereus*, *B. fastidiosus*, "*B. macroides*," *B. megaterium*, *B. psychrophilus*, and *B. schlegelii* present a square lattice, while strains of *B. stearothermophilus* can be obtained that individually have one of the three types (Claus and Berkeley, 1986).

As with S-layers of other bacteria, their function in *Bacillus* is unknown. However, since it has been demonstrated that the S-layer can physically mask the negatively charged peptidoglycan sacculus in *B. stearothermophilus* and prevent autoagglutination, it has been postulated that the layer may play a key role in bacteria-metal interactions (Sleytr and Messner, 1988).

Capsules

The capsule (a homopolypeptide of D-glutamic acid) of *B. anthracis* as a virulence factor has been studied extensively (see The Genus *Bacillus*—Medical in this Volume). Other bacilli, such as *B. subtilis*, *B. megaterium*, and *B. licheniformis*, possess capsules containing the homopolypeptide of D- or L-glutamic acid as well (Makino et al., 1989). Some *Bacillus* species, e.g., *B. circulans*, *B. mycoides*, and *B. pumilus*, produce carbohydrate capsules. For example, *B. circulans* forms an extracellular polymer consisting of glucose and glucuronic acid (Claus and Berkeley, 1986). In the case of *B. megaterium* a heteropolysaccharide composed of D-glucose, D-xylose, D-galactose, and L-arabinose has been found in one strain (Cassity and Kolodziej, 1984).

Flagella

Most *Bacillus* species possess peritrichous flagella. Although some use has been made of H-antigens in setting up serotyping schemes, they have not been widely adopted (Claus and Berkeley, 1986). Chemotaxis has been studied extensively in *B. subtilis* (Ordal and Nettleton, 1985).

Cell Walls

Almost all *Bacillus* species tested have vegetative cell walls made up of peptidoglycan containing *meso*-diaminopimelic acid (*m*-DAP). The exceptions (*B. sphaericus* and the related species, *B. pasteurii* and *B. globisporus*) contain lysine instead (Bartlett and White, 1985). But even those species, as all others, contain *m*-DAP in the peptidoglycan of their spore cortex. Cell wall turnover in Gram-positive bacteria, particularly *Bacillus* species which have been useful models, has been reviewed by Doyle et al. (1988). In addition to peptidoglycan in the cell wall, all *Bacillus* species contain large amounts of an anionic polymer, such as teichoic acid (a glycerol or ribitol-based polymer joined together by phosphodiester linkages to form a flexible linear strand) or teichuronic acid (uronic acid-based polymer) which are bonded to muramic acid residues. The type of this anionic polymer present depends on the levels of phosphate and magnesium in the growth medium. The glycerol teichoic acids vary a great deal between *Bacillus* species and within species. For example, *B. subtilis* can contain either glucosyl α or β (1→2) glycerol or glucosyl α (1→6) galactosyl α (1→1 or 3) glycerol), while *B. licheniformis* contains galactosyl α (1→2) glycerol. However, they are joined to the peptidoglycan through a common linkage disaccharide, acetylmannosaminyl(1→4)*N*-acetylglucosamine (Kaya et al., 1984).

As in other Gram-positive bacteria, lipoteichoic acids are found associated with most of the cell membranes of *Bacillus* species. These compounds are involved in the synthesis of wall

teichoic acids as regulators of autolytic activity and as scavengers of bivalent ions. Those so tested in *Bacillus* fall into three groups based on the presence or absence of *N*-acetylglucosamine branches in the backbone chains of their lipoteichoic acids—hydrophilic poly (glycerol-phosphate) chains and hydrophobic gentiobio-syldiacylglycerol anchors (Iwasaki et al., 1989). Group A is made up of strains of *B. subtilis*, *B. licheniformis*, and *B. pumilus*; group B, other *B. subtilis* strains and *B. cereus*, and group C, *B. polymyxa* and *B. circulans*.

Macrofibers

Macrofibers are multicellular and multistranded structures, hundreds of micrometers in length, produced by autocatalytic mutants of *B. subtilis* (Mendelson, 1978). These left- or right-handed helical structures have been used to study cell wall structure and growth. They are thought to reveal cell-surface molecular organization and force interactions in the cell wall not readily elucidated in the wild type, single-celled organism. The establishment and maintenance of macrofiber structure is influenced by both genetic and physiological factors (Briehl and Mendelson, 1987; Surana et al., 1988).

Membranes

The membranes of *Bacillus* species have been studied extensively because of their intrinsic interest; as a model of membrane structure in Gram-positive cells; with regard to their role in sporulation and germination; with respect to explanations for thermophily; and other reasons. For example, one explanation for the ability of thermophilic microorganisms such as *B. stearo-thermophilus* to grow at high temperatures is that the physical properties of the membrane are changed due to changes in the lipid composition in response to growth temperature (Gould, 1983).

There is great diversity in the range and type of lipids in *Bacillus* membranes (see review by Minnikin and Goodfellow, 1981) and wide variation in the fatty acids are found. The main phospholipids present are phosphatidylglycerol, diphosphatidylglycerol, and phosphatidyletha-nolamine; however, others are found as well. The major isoprenoid quinones are menaquinone, and most species contain menaquinones with seven isoprenoid units (MK-*7*). *B. acidocaldarius* is the exception and possesses MK-*9* (Minniken and Goodfellow, 1981).

Two-dimensional polyacrylamide gel electrophoresis (PAGE) has been used to attempt to resolve all *B. subtilis* membrane polypeptides (see Shohayer and Chapra [1985] for one such study). Several membrane enzymes have been isolated and characterized, such as the lactate, malate, glycerate-3-phosphate, NADH, and succinate dehydrogenases.

Because of interest in synthesis and modification of the peptidoglycan layer, much attention has been paid to penicillin-binding proteins. Six have been found in *B. subtilis*, but one of the most penicillin-sensitive binding proteins, number 4, has been found to be absent from *B. subtilis* 168 (Buchanan, 1987).

Genetic Studies

The discovery of transformation in *B. subtilis* strain 168 by Spizizen (1958) was largely responsible for focusing attention on the genetics of the genus *Bacillus*. Strain 168 is thought to be a derivative of the type strain *B. subtilis* Marburg (see Hemphill and Whiteley, 1975), and is one of a relatively few bacilli in which competence for DNA uptake has been found to occur as a natural part of the life cycle. As a consequence of Spizizen's discovery and the later isolation of generalized transducing phages, our knowledge of the chromosomal organization of *B. subtilis* is second only to that of the enteric bacteria. (About one-half as many genetic markers are known in *B. subtilis* as in *Escherichia coli*.) Furthermore, the identification of numerous genes affecting sporulation in *B. subtilis* is providing a means for analyzing this complex developmental program, which is largely unique to the Gram-positives.

Transformation

The establishment of a competent state in broth culture is most efficiently brought about in a minimal salts medium (Anagnostopoulos and Spizizen, 1961; Bott and Wilson, 1967). As the bacteria enter stationary phase in this medium, a maximum of 20% become competent, with a 1 to 2% transformation frequency for a given marker. The development of competence is associated with a reduction in macromolecular synthesis that is initiated well before cells are transformable. Competent bacilli are relatively metabolically latent, and have a lower buoyant density compared to noncompetent bacteria (Dooley et al., 1971; Hadden and Nester, 1968). Although the period of competence overlaps the time in which sporulation is initiated, the two forms of physiological differentiation are thought not to be connected. Different media are preferred for the two processes and commitment to competence is reversible. Several lines of research suggest that

the establishment of competence is coincidental with, and perhaps induces, a DNA-repair system analogous to the SOS regulon in *E. coli* (Love et al., 1985).

The development of the capacity of *B. subtilis* to take up DNA is associated with the appearance of several novel cellular proteins. Among these is a 75-kDa protein complex which has been isolated from the membrane of competent cells (Smith et al., 1985). The complex consists of two subunits of 18 kDa (polypeptide A) and 17 kDa (polypeptide B). Mutants lacking subunit A do not bind DNA to the cell surface whereas those deficient in B are defective in DNA entry. The 75-kDa protein complex also has nuclease activity that is probably associated with polypeptide B. A nuclease is expected, because it is known that one strand of transforming DNA is hydrolyzed in the process of entering the cell, resulting in a single-stranded product on the cytoplasmic side of the membrane (Davidoff-Abelson and Dubnau, 1973a, 1973b). Competence factors that interact with the membrane have also been reported in *B. stearothermophilus* (Streips and Welker, 1971). *B. subtilis* cells show little specificity with regard to DNA uptake and may be transformed with homologous chromosomal DNA, plasmid DNA, or transfected with bacteriophage DNA.

There is evidence that strands of transforming DNA enter the cell at sites on the membrane where the chromosomal DNA is attached (teRiele et al., 1984). The penetrating DNA, now reduced to a single-strand form, is then brought in contact with the homologous region of the recipient chromosome. This step is probably mediated by the 45-kDa protein product of the *B. subtilis recE* gene and results in a complex in which the transforming DNA begins to displace one strand of the chromosomal DNA while hydrogen-bonding to the complementary strand. A continuation of the displacement reaction allows pairing and integration of several thousand bases of transforming DNA into the chromosome, while an equivalent amount of the homologous strand is removed and degraded.

Considerable thought has been given to the question of whether transformation is associated with genetic exchange in natural populations of bacilli (see Stewart and Carlson, 1986, for a review). The complexity of the transformation process with its requirement for unique competence factors appearing only at stationary phase suggests that the capacity to take-up exogenous DNA offers some selective advantage in the evolution of these bacteria. The fact that competence occurs only late in the growth cycle probably means the system is not designed to obtain DNA as a nutrient. Ephrati-Elizur 1968 found that *B. subtilis* cells excrete high-molecular-weight DNA into liquid culture as they grow. Under natural conditions this could be the source of donor DNA. Graham and Istock (1978) demonstrated that genetic exchange, thought to be mediated by transformation, occurs at high frequency between genetically labeled strains of *B. subtilis* in soil. Also, transformation frequencies in cultures in which the bacteria are allowed to attach to sand grains are much higher than in the standard liquid culture procedure (Lorenz et al., 1988).

Generalized Transduction

Bacteriophage capable of mediating generalized transduction have been reported in many species of *Bacillus* including *B. subtilis*, *B. cereus*, *B. megaterium*, *B. thuringiensis*, *B. anthracis*, and *B. stearothermophilus*. Thus, transduction offers an immediate advantage for genetic analysis over transformation in that it is applicable to more strains of these bacteria. In addition, some phages transduce fragments of DNA much larger than can be transferred via transformation, and this facilitates linking distant markers. PBS1, a bacteriophage that infects *B. subtilis* 168, can incorporate 5 to 10% of the bacterial chromosome in a single virion particle, and was instrumental in constructing the complete circular chromosomal map of *B. subtilis* (Lepesant-Kejzlarova et al., 1975). On the other hand, small DNA molecules such as those of some plasmids are not efficiently packaged in large phages, but can be transduced by a variety of small bacteriophages (Canosi et al., 1982). Some generalized transducing phages have relatively broad host ranges and can transfer plasmids between different species of bacilli (Ruhfel et al., 1984).

Little is known about the mechanism by which transducing particles are formed. PBS1 appears to package bacterial DNA randomly. There is little evidence for packaging sites as are indicated in *Salmonella* phage P22 (Jackson et al., 1978; Schmieger, 1982, 1984). A *pac* site has been located in the genome of the small generalized transducing phage SPP1. However, its relevance, if any, to packaging transducing DNA is not clear (Deichelbohrer et al., 1982, 1985).

The process by which transducing DNA becomes incorporated into the recipient bacterial chromosome differs from that discussed earlier for transformation. In transduction, the donor DNA is thought to enter the infected cell as double-stranded DNA which then synapses with the homologous region of the recipient chromosome. Incorporation of the transducing fragment presumably results from a double

crossover between the two DNA molecules. In support of this model, mutants of *B. subtilis* deficient in recombination functions have a reduced capacity to be transduced, while they can be transformed (Dodson and Hadden, 1980).

Other important *Bacillus* generalized transducing phages include CP15, a phage originally shown to transduce *B. cereus* (Thorne, 1968) but which can transfer plasmids between *B. cereus*, *B. thuringiensis*, and *B. anthracis* (Ruhfel et al., 1984). Bacteriophage MP13 has been important in mapping the chromosome of *B. megaterium* (Vary et al., 1982), and TP-13 and TP18 have been similarly used in genetic studies of *B. thuringiensis* (Barsomian et al., 1984).

Specialized Transduction

Specialized transduction has been reported in several *B. subtilis* phages including π105 (Shapiro et al., 1974), SPβ (Zahler et al., 1977), and π3T (Odebralski and Zahler, 1982) and *B. amyloliquefaciens* phage H2 (Zahler et al., 1987). The most carefully examined of these is SPβ, a temperate bacteriophage that is normally carried as a prophage in *B. subtilis* 168 (Warner et al., 1977). SPβ can transduce markers proximal to its normal attachment site *attB* SPβ between the markers *ilvA* (threonine dehydratase) and *kauA* (ketoacid uptake) located near the terminus of chromosomal replication. In addition, SPβ prophage will insert at a variety of aberrant positions when it lysogenizes mutants of *B. subtilis* lacking *attB* SPβ. Induction of these lysogens gives rise to specialized transducing phages carrying genetic markers near the novel sites of integration (see Zahler, 1982, for a review). The gene order of SPβ prophage is a circular permutation of that present in the phage DNA (Spancake et al., 1984). Specialized transducing particles are thought to originate from errors in excision when the prophage is induced. The crossing-over event that leads to reformation of a circular phage DNA during induction may be displaced, resulting in removal of a portion of the bacterial DNA contiguous to the right or left boundaries of the prophage. Encapsidation of such hybrid DNA produces a specialized transducing particle.

Two types of transductants are recognized in the SPβ system (Zahler, 1982). The bacterial portion of the transducing phage DNA may undergo recombination with and replace the homologous region of the genome of the recipient. This so-called "replacement transduction" is similar to generalized transduction, except that only a limited number of genetic markers are involved. On the other hand, the infecting phage-bacterial DNA may incorporate as a prophage to produce a bacterium diploid for the *B. subtilis* genes associated with the prophage. Such "addition transductions" are most likely to occur if the recipient bacterium is already lysogenic for SPβ. The resulting merozygote may be used in complementation studies if different alleles are present on the prophage and bacterial chromosomes.

Conjugative Plasmids

Fertility plasmids capable of bringing about their own transfer from one bacterium to another have been described in several species of *Bacillus*. The capacity to produce the insecticidal delta toxin crystal protein in *B. thuringiensis* is encoded in large plasmids. Gonzalez et al. (1982) found that three strains of this bacterium transmitted the crystal-protein phenotype to *B. thuringiensis* variants which had lost the plasmid. Moreover, these plasmids could also be transferred to *B. cereus* and yielded transcipients that produce crystal protein. Battisti et al. (1985) reported the transfer of plasmids pXO11 and pXO12 from *B. thuringiensis* to *B. anthracis* and *B. cereus*. The transcipients, in turn, became effective donors, and in the case of those inheriting pXO12, also acquired the ability to produce parasporal crystals. Strains of *B. anthracis* that acquire plasmid pXO12 can subsequently mobilize and transfer nonconjugative plasmids present in the same cell. Using this system, the tetracycline resistance plasmid pBC16, the *B. anthracis* toxin plasmid pXO1, and the capsule plasmid pXO2 have been transmitted to *B. anthracis* and *B. cereus* recipients lacking these plasmids (Green et al., 1989). The small plasmid pBC16 is transferred at high frequency without direct interaction with pXO12; such transfer of a nonconjunctive plasmid is called donation. The large *B. anthracis* plasmids are apparently transferred by conduction. The latter involves formation of cointegrative molecules in the donor, and resolution of the cointegrates into pXO12 and the respective *B. anthracis* plasmid in the recipient. Cell-to-cell contact is necessary for plasmid transfer and is resistant to DNase, but little is known about the mechanisms or conjugative structures that may be involved.

A strain of *B. subtilis* (natto) has been found which carries a 55-kb self-transmissible plasmid (pLS20), which can be transferred to closely related strains and to restriction-deficient strains of *B. subtilis* (Koehler and Thorne, 1987). This plasmid also promotes the transfer of the tetracycline-resistance plasmid pBC16 from *B. subtilis* (natto) to a wide variety of *Bacillus* species including *B. anthracis*, *B. megaterium*, and *B. subtilis*. This is a much broader range of conjugative transmission than has been observed with the *B. thuringiensis* plamid. However, none of

the conjugative plasmids have been found to mobilize and transfer chromosomal markers as is observed with the F plasmid of *E. coli*.

In addition to the naturally occurring transmissible plasmids of *Bacillus*, Christie et al. (1987) have identified a conjugative transposon (Tn925) which transfers from *Streptococcus faecalis* to *B. subtilis*.

Bacteriophages

Bacteriophages that infect *Bacillus* are common in soil. In addition, many strains of this genus are naturally lysogenic for one or more prophages. The most extensively studied *Bacillus* phages are those associated with *B. subtilis*, and these have been reviewed by Hemphill and Whiteley (1975), Rutberg (1982), and Zahler (1988).

With some exceptions, *Bacillus* phages have relatively narrow host ranges, probably at least in part because of restriction systems that make phage grown in one host incompatible with another strain (Ando et al., 1982). With the exception of the phages of *B. subtilis*, no scheme of classification has been adopted to organize the phages of this genus. Therefore, the bacteriophages described here will be grouped according to life cycle.

Temperate Bacteriophages

Most strains of bacilli that have been carefully examined have been found to release phage particles. These are of two types: defective phages that can kill but do not productively infect other strains (see "Defective Bacteriophages," this chapter), and those that grow on and lysogenize new host bacteria. *B. subtilis* 168 is lysogenic for phage SPβ and also releases defective phage PBSX. As an extreme, *B. thuringiensis* subsp. *aizawai* is polylysogenic for five unique temperate phages (Reynolds et al., 1988). Temperate phages are easily obtained from nature. If samples of soil are placed in broth and the mixture heated 10 min at 80°C, most free phage and non-sporeforming bacteria are destroyed. When the culture is allowed to incubate several hours and

subsequently treated with mitomycin C, temperate phages are induced and released into the medium. (Some investigators inoculate the broth with the *Bacillus* strain of interest to enrich for phage, and then add mitomycin C.) The phages may then be recovered by filtering the solution and plating on an appropriate indicator.

Dean et al. 1978 have divided the temperate phages of *B. subtilis* and closely related species into four groups based upon serology, immunity, and physical characteristics (Table 9); the defective phages may be considered a fifth class. Several group III phages including *B. subtilis* phages SPβ and π3T and *B. amyloliquefaciens* phage H2 can mediate specialized transduction. Group I phage π105 also transduces genes close to its prophage attachment site (Shapiro et al., 1974). In addition, π105, SPβ, ρ11, and others have been used as cloning vehicles, mostly for *B. subtilis* genes (see Zahler, 1988, for a review).

Temperate bacteriophages often alter the biochemistry or phenotype of lysogenic bacteria and several examples of such prophage conversion have been observed in *B. subtilis*. Strains of this bacterium lysogenic for SPβ release a bacteriocin-like substance called betacin (Hemphill et al., 1980) which kills some *Bacillus* strains that do not harbor the SPβ prophage. Most group III bacteriophages including π3T, ρ11 and Z (but not SPβ) contain the structural gene for thymidylate synthetase, and express this gene continuously in lysogens. Stains of *B. subtilis* lysogenic for SP02 cannot be productively infected with virulent phage π1 (Rettenmier and Hemphill, 1974), and bacilli lysogenic for SPβ are protected by a similar interference system active against π1*m* (Rettenmeir et al., 1979).

Defective Bacteriophages

Many species of *Bacillus* including *B. subtilis*, *B. amyloliquefaciens*, *B. pumilis*, and *B. laterosporus* release defective phages whose presence is revealed by their bactericidal activity against other strains or species of this genus (Hemphill and Whiteley, 1975; Steensma et al., 1978; Zahler, 1988.) For example, *B. subtilis* strain 168 releases a defective phage called

Table 9. Major groups of temperate *B. subtilis* phages.

Group	Example	DNA size (kb)	Virion dimensions (nm)		Other members of group
			Head	Tail	
I	φ105	40	52 × 52	10 × 220	ρ14
II	SPO2	40	50 × 50	10 × 180	—
III	SPβ	126	72 × 82	12 × 358	φ3T, ρ11, Z, SPR
IV	SP6	53	61 × 61	12 × 192	—
V	PBSX	13	45 × 45	20 × 200	PBSZ

Modified from Dean et al. (1978) and Zahler (1988).

PBSX, which kills the cells of *B. subtilis* strain W23. Electron microscopic examination of the culture supernatant of strain 168 reveals typical phage particles. However, the PBSX virions cannot replicate and produce plaques; rather, they act much like a bacteriocin. *B. subtilis* strain W23, in turn, releases defective phage PBSZ, which has a bacteriocin activity against strain 168.

Despite being defective, these phages act much like other temperature viruses. Although small numbers are typically present in the culture supernatant, lysogens may be induced with UV light or mitomycin C and lyse and release large numbers of particles. (The situation is often confusing, however, because many bacilli including *B. subtilis* 168 are simultaneously lysogenic for one or more nondefective temperate phages.) Virions of PBSX are unmistakably phage-like in structure. They have a rather small head (45 nm in diameter) and a cylindrical, contractile tail 18 by 200 nm. The genes for structural proteins of PBSX have been mapped to a chromosomal position between the markers *metA* and *metC* (Thurm and Garro, 1975). PBSX and presumably other phages of this group are defective for more than one reason. First, the particles do not contain an identifiable phage DNA. Instead, PBSX packages randomly selected 13-kb fragments of bacterial chromosomal DNA (Anderson and Bott, 1985). Second, although PBSX virions attach efficiently to susceptible strains and the tail appears to contract, the DNA in the phage heads is not injected (Okamoto et al., 1968).

Lytic Bacteriophages

Lytic bacteriophages that infect *Bacillus* are also common in soil and water. Again, the most detailed studies are of phages that infect *B. subtilis* or closely related species (see Hemphill and Whiteley, 1975, for a review.) Although many of these viruses are intrinsically interesting, two groups of subtilis phages have been the focus of studies on the regulation of viral transcription (see Geiduschek and Ito, 1982). The first class, which includes SP01 and SP82, is distinguished by the replacement of thymine by hydroxymethyluracil (hmU) in the viral DNA. The second class includes $\pi 29$ and relatives, which are very small, linear double-stranded DNA phages.

SP01 and SP82 have very complex temporal programs of transcription involving at least three phases of gene expression designated early, middle, and late. Early gene expression starts within 1 min of infection, and is thought to use the host RNA polymerase. Middle genes are activated about 4 min after infection, and the expression of late genes (which are turned on asynchro-

nously) is first detected 10 min into the latent period. Initiation of transcription of the middle and late genes involves structural modifications of the *B. subtilis* RNA polymerase, such that the enzyme can recognize unique middle and late phage promoters that are not used by the unmodified polymerase (Tarkington and Pero, 1979). The product of at least one SP01/SP82 gene is thought to associate with the enzyme to activate middle genes (Hyde et al., 1986), and at least two other phage-encoded polypeptides appear to modify the polymerase to synthesize late mRNAs (Fox, 1976; Tjian and Pero, 1976). Other viral genes associated with late transcription have been identified, but these may be required to initiate DNA replication, which is also involved in transcriptional control. Even as new mRNAs are made, transcription of at least some early and middle genes is repressed at characteristic times in the latent period of SP82 and SP01. Proteins that bind to phage DNA and block RNA synthesis have been isolated from infected cells, but the actual mechanism of repression is not clear. Although not directly related, the study of gene expression in these subtilis phages has influenced the investigation of somewhat analogous changes in RNA polymerase structure associated with sporulation.

Transcription of the $\pi 29$ genome also involves early and late mRNAs, which in this case are transcribed from different strands of the viral DNA. Early RNA is transcribed from the light (L) and late RNA from the heavy (H) strand. In vitro, *B. subtilis* RNA polymerase synthesizes early mRNA from as many as eight promoters (Mellado et al., 1988). At least a portion of the host RNA polymerase is also used for synthesis of late RNA, which is apparently initiated from a single promoter called A3. The latter has a −35 region that differs somewhat from the consensus −35 region of *B. subtilis* promoters (Vlcek and Paces, 1986). Transcription from A3 requires protein P4 encoded by $\pi 29$ gene 4 (Mellado et al., 1988).

Pseudotemperate Phages

Pseudotemperate bacteriophages are lytic phages that establish a relatively long-term association with bacteria that mimics true lysogeny (see Hemphill and Whiteley, 1975). Following adsorption and penetration, further advancement of the viral development cycle is often delayed; some infected cells continue to grow, divide, and even sporulate. A portion of the daughter cells may even be cured, and about one-half the spores produced from such cultures do not contain viral DNA. Pseudotemperate bacteriophages characteristically produce turbid plaques, and "pseudolysogens" obtained from

these plaques may be subcultured. The pseudolysogens apparently consist of a balance between infected cells in a delayed latent period, bacteria containing maturing phage, and uninfected bacilli.

Included among the pseudotemperate phages are several of the best-known transducing phages of *Bacillus* such as *B. subtilis* phages PBS1, SP10, and SP15 and *B. thuringiensis* phage TP-13. The genomes of these viruses often contain modified DNA. For instance, the chromosome of PBS1 has a complete replacement of thymine by uracil (Takahashi and Marmur, 1963) and SP15 DNA substitutes 5-(4′-5′-dihydroxylpentyl)-uracil for thymine (Brandon et al., 1972).

Phage PBS1 (also referred to as PBS2, a clear-plaque mutant) and phage PMB12 carry out their entire development cycle in the presence of rifampin, an antibiotic that inhibits RNA synthesis by interacting with the bacterial RNA polymerase (Rima and Takahashi, 1974; Bramucci et al., 1977). In the case of PBS1 (PBS2), the resistance to rifampin is explained in part by the synthesis of a new phage-induced RNA polymerase that transcribes the viral late functions (Clark et al., 1974). Genetic evidence suggests that early RNA synthesis in PBS1 may be resistant to the drug because the virus, perhaps using a virion protein, induces a modification in the bacterial RNA polymerase that converts the latter to a resistant form (Osburne and Sonenshein, 1980).

One of the most intriguing phenomena associated with bacteriophage PMB12 is its capacity to convert pseudolysogens of certain sporulation-negative (*spo*) mutants of *B. subtilis* to a Spo+ phenotype (Kinney and Bramucci, 1981). Conversion has been observed in several stage O mutants, as well as in *B. subtilis* variants deficient in sporulation due to changes

in bacterial RNA polymerase or 30S ribosomes. At least three PMB12 genes are involved in spore conversion. The products of these genes apparently interact with host-cell pathways at the earliest stages of sporulation. Conversion of spore-negative mutants has also been observed with *B. pumilis* phage PMB1 (Bramucci et al., 1977) and numerous other phages which infect this species (Keggins et al., 1978). Bacteriophage TP-13 similarly converts oligosporogenic, acrystalliferous mutants of *B. thuringiensis* to spore-positive, crystal-positive phenotype (Perlak et al., 1979). This phage also mediates generalized transduction of large DNA fragments.

Plasmids

Plasmids are of widespread occurrence in the genus *Bacillus* and have been found in most species that have been screened. The vast majority are cryptic plasmids, that is, their presence has not been correlated with any unique property of the bacterial host. Although efforts have been made to analyze the relationship between different plasmids in a few species by comparing DNA-restriction fragment profiles, there is no accepted scheme for systematically classifying plasmids within the bacilli. Further confusing the study of plasmids in *Bacillus* is the fact that some of those used as vectors for gene cloning, such as pUB110, were actually derived from *Staphylococcus aureus* (Gryczan et al., 1978).

As shown in Table 10, some of the *Bacillus* plasmids do confer a recognizable phenotype to cells which carry them, such as antibiotic resistance, synthesis of toxins, unique metabolic activities, and transconjugation. Because of the medical and economic importance of their hosts, the plasmids conferring virulence in *B. anthracis*

Table 10. *Bacillus* plasmids.

Bacterium	Plasmid	DNA size (kb)	Phenotype associated with plasmid	Reference
B. anthracis	pXO1	168	Exotoxin (lethal factor, edema factor, protective antigen)	Mikesell et al., 1983 Tippetts and Robertson, 1988
	pXO2	85.6	Capsule	Green et al., 1985
B. cereus	pBC7	69	Bacteriocin	Bernhand et al., 1978
	pBC16	4.3	Tetracycline resistance	Bernhand et al., 1978
B. pumilus	pBL10	6.8	Bacteriocin	Lovett et al., 1976
B. subtilis	pIM13	2.2	Erythromycin resistance	Mahler and Halvorson, 1980
B. subtilis (natto)	pLS19	5.4	Polyglutamate production	Hara et al., 1982
	pLS20	55	Self-transmissible plasmid, which also promotes transfer of other plasmids	Koehler and Thorne, 1987
B. thuringiensis	pXO12	112.5	Production of insecticidal crystal protein, and is a self-transmissible plasmid, which can co-transfer unrelated plasmids	Green et al., 1989
Bacillus species (thermophilic)	pTB19	26	Kanomycin and tetracycline resistance	Imanaka et al., 1981
	pTB20	4.3	Tetracycline resistance	Imanaka et al., 1981

and *B. thuringiensis* have been of increasing interest. Virulence in *B. anthracis* requires the production of a capsule composed of poly-D-glutamic acid and an exotoxin consisting of three components: protective antigen, lethal factor, and edema factor. The capacity to produce the capsule is associated with plasmid pXO2 (Green et al., 1985), and the constituents of the toxin are encoded in plasmid pXO1 (Mikesell et al., 1983). Strains losing either plasmid are rendered avirulent. With the help of fertility plasmid pXO12 derived from *B. thuringiensis*, both pXO1 and pXO2 have been transferred to bacilli lacking these plasmids (Green et al., 1989). In the case of pXO1, cured strains of *B. anthracis* have been restored to toxin production (Thorne, 1985). The association of the toxin with plasmids is of historical interest. The original Pasteur vaccine strains of *B. anthracis* were produced by subculturing this bacterium at high temperatures which are now known to inhibit replication of pXO1. Indeed, two existing Pasteur strains lack this plasmid (Mikesell and Vodkin, 1985).

The genes for delta-exotoxin crystal proteins of *B. thuringiensis* strains are carried in large plasmids (see The Genus *Bacillus*—Insect Pathogens in this Volume). These plasmids are somewhat divergent in size and the crystal proteins produced by *B. thuringiensis* strains differ in serology and toxicity to various insects (Höfte and Whiteley, 1989). Moreover, several of these plasmids, such as pXO12 of *B. thuringiensis* subsp. *thuringiensis* 4042, (Reddy et al., 1987; Green et al., 1989) have been shown to mediate conjugal transfer of themselves and other plasmids (see "Genetic Studies" this chapter).

The production of the parasporal body containing the crystal protein toxin is coordinated with sporulation; crystal protein gene expression begins about stage II (see Whiteley and Schnepf, 1986, for a review). Changes in transcription during sporulation are associated with modifications of RNA polymerase which enable the enzyme to recognize sporulation-specific promoters (see "Sporulation", this chapter). It has been suggested the activation of the crystal protein genes is also regulated by polymerase changes. DNA sequence analysis of the 133-kDa crystal protein gene from *B. thuringiensis* subsp. *kurstaki* HD-1-Dipel revealed two overlapping promoters, one used early in sporulation (BtI) and the other (BtII) activated midway through development (Wong et al., 1983). Comparison of these promoters with those of the carefully studied *B. subtilis* system show little sequence consensus with promoters that bind vegetative RNA polymerase. Brown and Whiteley (1988) have isolated an RNA polymerase from strain HD-1-Dipel that directs transcription in vitro from the BtI promoter and also transcribes crystal protein

genes from two other strains of *B. thuringiensis*. This polymerase contains a unique 35-kDa sigma subunit that is different from the major sigma subunit present in vegetative cells of *B. thuringiensis*. In addition, the presence of two regions of hyphenated dyad symmetry near the BtI and BtII promoters suggests that binding of other regulatory proteins in this region could be associated with gene expression.

Transcriptional Regulation in Biosynthetic and Catabolic Pathways

Bacillus species demonstrate great catabolic and biosynthetic versatility. It is only recently, however, that significant progress has been made in understanding how synthesis of enzymes is regulated. Again, most studies have concentrated on *B. subtilis* and closely related species.

Control of Biosynthetic Gene Expression

B. subtilis can be grown in minimal salts media with glucose as carbon source. Thus, this bacterium must have the capacity to synthesize de novo all amino acids, nucleotides, etc. As in the enteric bacteria, the structural genes for enzymes of common biosynthetic pathways are clustered on the *B. subtilis* chromosome (see Zalkin and Ebbole, 1988, for a review.) Such operon-like organization has been observed for the genes of the tryptophan, arginine, and isoleucine-valine-leucine synthesis. In addition, the sequences encoding the enzymes for purine (*pur* genes) and pyrimidine (*pyr* genes) occur in multicistron clusters in *B. subtilis* (Ebbole and Zalkin, 1987; Lerner et al., 1987), which is not the case in *E. coli*. Gene clustering is associated with coordinate control of gene expression at the level of transcription by the products of the pathways. The *trp*, *arg*, and *ilv* operons are repressed, respectively, by tryptophan, arginine, and leucine. The *pur* genes are repressed by adenine and guanine nucleotides and *pyr* by pyrimidines.

It does not appear that regulation of the biosynthetic operons is mediated primarily through repressors, although the *arg* pathway may be an exception (Smith et al., 1986). Rather, the favored means of control appears to be termination/antitermination (attenuation) mechanisms which affect the elongation of nascent mRNA molecules. In several operons studied, the 5′-end of the mRNA contains a leader of 100 to 300 nucleotides which does not encode part of the first structural gene. Within this leader is a region that can fold into two mutually exclusive secondary structures, one of which is a procaryote transcription termination signal. The other con-

figuration is an antiterminator stem-and-loop (hairpin) which allows full-length message to be made. Control then resides in changing the ratio of termination vs. antitermination secondary structure.

In enteric bacteria, the leader sequence of amino acid biosynthetic gene clusters includes an open reading frame (ORF) for a short peptide, which is generally enriched in codons for the amino acid synthesized by that pathway. The formation of termination vs. antitermination hairpins is modulated by the rate at which the leader polypeptide is synthesized. In the *E. coli trp* operon, for example, the peptide is made slowly if intracellular levels of tryptophan are low, and the antitermination structure is generated. The converse is true if tryptophan levels are high. In *B. subtilis* those leader sequences that have been analyzed do not contain an ORF. Moreover, transcription of *pur* and *pyr* gene clusters is also thought to be controlled by termination/antitermination, and it is unlikely these operons could be regulated by a mechanism precisely analogous to the *E. coli* system.

In the *B. subtilis trp* operon, attenuation is regulated in part by a *trans*-acting factor thought to be the product of the methyltryptophan resistance locus *mtr* (Shimotsu et al., 1986). Mutations in this gene result in constitutive expression of the *trp* genes. It is likely that the *mtr* product, when activated by tryptophan, binds to the leader region of the nascent mRNA in such a manner as to prevent formation of the antiterminator structure, thus favoring termination. In addition, binding of tryptophan-activated *mtr* gene product to already completed mRNA molecules is thought to inhibit the initiation of translation, thereby further reducing the production of *trp* enzymes (Kuroda et al., 1988).

Transcriptional regulation of the *pur* operon of *B. subtilis* shows some similarities to that of the *trp* system, but may be even more complicated. Synthesis of inosine monophosphate (IMP) is repressed by both adenine and guanine nucleotides, but apparently by different mechanisms. The latter are thought to promote premature cessation of transcription at a rho-independent termination site in the leader sequence, while adenine nucleotides appear to repress transcription initiation (Zalkin and Ebbole, 1988). It is thought likely that when purine levels are high, a guanine nucleotide activates a regulatory molecule, which in turn binds to the leader region of the nascent mRNA and blocks formation of the antiterminator secondary structure.

The products of polycistronic mRNAs are sometimes subunits in a common enzyme complex. In these instances, the polypeptides are required in 1:1 stoichiometry. Sequence analysis of the *trp* and *pur* operons shows that both are characterized by many gene overlaps; that is, the 3′-end of one coding sequence overlaps with the 5′-end of a contiguous downstream coding sequence (Henner et al., 1984; Ebbole and Zalkin, 1987). The overlaps are thought to result in "translational coupling" in which synthesis of one polypeptide from a polycistronic mRNA is at least partially dependent on the translation of the contiguous upstream gene. In some instances, this coupled translation is thought to result in 1:1 stoichiometric synthesis of the products of the overlapping genes.

Control of Catabolic Gene Expression

Members of the family Bacillaceae catabolize a wide variety of simple and complex organic compounds, including mono-and disaccharides, and polysaccharides such as starch which are partially digested by extracellular enzymes. As in the enteric bacteria, the structural genes for enzymes involved in degradation of these substrates are often clustered on the *Bacillus* chromosome. All operons so far studied are transcribed from σx^A promoters, and control of gene expression involves repressors and termination/antitermination systems (see Klier and Rapport, 1988, for a review).

The pathway involved in gluconate catabolism in *B. subtilis* is apparently regulated by negative control mediated by a repressor. Two inducible enzymes are required: the transport protein gluconate permease and gluconate kinase which phosphorylates gluconate. The genes for these functions, *gntP* and *gntK*, respectively, are part of a four cistron operon *gntR-gntK-gntP-gntZ*. Mutations that inactivate *gntR* are constitutive. This gene encodes a 243 amino acid protein thought to inhibit transcription by binding near the promoter located 40 bp upstream from *gntR* (Fujita and Fujita, 1987). Curiously, there is a five base overlap between the coding sequence of *gntR* and *gntK* suggesting there may be a translational coupling between these two proteins (Fujita et al., 1986). The genes required for the growth of *B. subtilis* on xylose polymers are also organized in a five cistron regulon, in which one of the genes, *xylR,* codes for a repressor that regulates expression of the entire gene cluster (Hastrup, 1988). Synthesis of the enzymes is induced by the presence of xylose in the medium. A repressor system has also been found to regulate the induction of penicillinase in *B. licheniformis* (Wittman and Wong, 1988).

Perhaps the most extensively studied catabolic system in the bacilli is that involved in sucrose catabolism. *B. subtilis* produces two β,D-fructofuranosidases, sucrase and levansucrase, after induction by sucrose. The former is an intracellular enzyme while the latter (which also cat-

alyzes the formation of the fructose polymer levan) is secreted (see Klier and Rapoport, 1988). The structural genes for sucrase, *sacA*, and that for levansucrase, *sacB*, are widely separated on the *B. subtilis* chromosome. The *sacA* gene is tightly linked to *sacP*, which is thought to be a membrane-associated protein that transports and phosphorylates sucrose. The *sacB* gene and the *sacA-sacP* cistrons are linked to regulatory loci, *sacR* and *sacT*, respectively. Mutations in either of the regulatory loci results in constitutive synthesis of the corresponding enzyme. The most extensive studies of the mechanisms of control have been made in *sacR*.

The *sacR* locus contains the *sacB* promoter. Between the transcription start site and the *sacB* coding sequence is a 199-bp region that is the target of several regulatory effectors of expression of the levansucrase gene (Aymerich et al., 1986). Part of this control is thought to involve a region of dyad symmetry that could form a transcription termination signal in the nascent mRNA. Deletion of this perspective terminator results in constitutive synthesis of levansucrase, and previously isolated constitutive *sacR* mutants map to this putative stem-and-loop structure (Shimotsu and Henner, 1986; Steinmetz and Aymerich, 1986). Messenger RNA synthesis is thought to stop at this terminator unless an antiterminator protein, presumably regulated by sucrose, interacts with the nascent RNA to prevent formation or to allow bypass of the loop. One candidate for the antiterminator maps to *sacS*, a genetic locus that affects both the synthesis of levansucrase and sucrase. The *sacS* locus consists of two cistrons, *sacY* and *sacX* (Aymerich and Steinmetz, 1987; Steinmetz et al., 1988; Zukowski et al., 1988). The *sacY* gene is thought to encode a sucrose-dependent antiterminator protein whose target probably is the region of secondary structure in *sacR*, where it presumably acts to prevent formation of the stem-and-loop. Deletion of *sacY* abolishes levansucrase synthesis. Current data suggest the *sacX* gene directs the production of a negative regulator of *sacY*; mutants in this cistron produce levansucrase constitutively.

The products of two other regulatory genes, *sacU* and *sacQ*, have been implicated in regulation of levansucrase synthesis. The target of the products of these genes also appears to be the *sacR* region, and they may serve to modulate the levels of gene expression (Shimotsu and Henner, 1986; Zukowski and Miller, 1986). *sacQ* encodes a 46 amino acid polypeptide which appears to stimulate the transcription of several secreted enzymes. Some mutations in *sacQ* lead to higher levels of expression of target loci, and genes thought to be similar to *sacQ* have been cloned from *B. licheniformis* and *B. amylolique-*

faciens. It now appears that the *sacQ* polypeptide is one of many small polypeptides which somehow regulate the transcription of degradative enzymes in the family Bacillaceae. Others may include the products of the *sacV*, *sin*, and *ptrR* genes (see Klier and Rapoport, 1988).

The enzymes for the histidase pathway responsible for the degradation of histidine are encoded in a cluster of four genes *hutH-hutU-hutI-hutG* in *B. subtilis*. Expression of this *hut* operon is induced by histidine and is also subject to catabolite repression. Between the σ^A promoter and the first gene, *hutH*, is a regulatory locus, *hutR*. The latter has been cloned and sequenced and found to contain two regions of possible regulatory significance (Oda et al., 1988). One is an ORF (ORF1) for a 151 amino acid protein that acts *trans* in heterozygotes to regulate the *hut* operon. A mutant having an amino-acid substitution in this protein has been isolated and found to be uninducible for the histidase pathway enzymes. This suggests that the ORF1 protein is a positive regulator in expression of the *hut* operon, although the possibility that the mutant is a superrepressor cannot be entirely excluded. The second regulatory region located between ORF1 and the first structural gene *hutH* (ORF2) is a region of dyad symmetry which could form a rho-independent terminator. It is possible that the product of ORF1, presumably activated by histidine, acts as an antiterminator to allow complete transcription of the *hut* operon.

The picture emerging from these and other studies suggests that regulation of catabolic pathways in *B. subtilis* involves a combination of repressors and positive controls through antitermination. It should be noted that in the attenuation systems observed in the biosynthetic pathways the leader sequences appear to be able to form two mutually exclusive secondary structures, one of which is the terminator. In the several leader sequences studied in catabolic pathways, only the terminator stem-and-loop is indicated. Thus, the putative antiterminator proteins in catabolic systems probably act by blocking formation of or allowing bypass of the terminator, rather than acting to favor one secondary structure over the other.

Catabolite Repression

Catabolic pathways in *Bacillus* are subject to catabolite repression by high levels of glucose and a variety of other rapidly metabolized substrates (Nihashi and Fujita, 1984). This phenomenon is of special interest in bacteria of this genus because sporulation is also repressed by glucose. In the enteric bacteria, catabolite

repression is mediated in part by the levels of cAMP and the interaction of this nucleotide with catabolite activator protein (CAP). However, cAMP apparently is not present in *Bacillus*, and thus could not be involved in catabolite repression in these organisms.

The promoters and associated control regions of many catabolic operons from *Bacillus* have been cloned and sequenced (Laoide et al., 1989; Melin et al., 1987; Oda et al., 1988). In some instances, these cloned regulatory systems, which for experimental purposes are sometimes fused to an indicator gene such as *E. coli lacZ*, continue to be subject to glucose suppression of transcription. Among the most thoroughly studied is the complex *amyR1-amyE*, the regulatory and structural genes for alpha-amylase of *B. subtilis*, and *amyR1-amyL*, the corresponding regulatory region and structural gene for alpha-amylase of *B. licheniformis*. Amylase synthesis is actually under two forms of regulation. One is a temporal control manifested by the fact that amylase genes are not fully induced until the onset of stationary phase. Temporal control may not occur in all *Bacillus* strains (Rothstein et al., 1986). In addition, in both *B. subtilis* and *B. licheniformis FDO2* production of alpha-amylase is repressed about 10-fold in glucose-containing cultures in early stationary phase, even when multiple copies of the complex are present on a plasmid (Laoide et al., 1989). Both forms of regulation are associated with the *amyR* regions. A plasmid-borne construct of *amyL* missing the promoter but including all transcribed sequences immediately adjacent and downstream from the promoter has been produced. When this fragment is attached at various distances from heterologous promoters, transcription continues to be subject to catabolite repression. This suggests the target of repression is not in the promoter itself or in regions upstream from it. One possibility is that a regulatory protein involved in mediating catabolite repression binds to a *cis*-acting site very close to the transcription start site, which in the case of *amyL* is only 29 to 31 nucleotides from the translation initiation codon (121 nucleotides for *amyE*). A search for consensus sequences downstream from several promoters of *B. subtilis* genes subject to catabolite repression has yielded a candidate sequence: 5′-ATTGTNA-3′ (Laoide et al., 1989). In the regulatory loci for *amyL* and *amyE*, this sequence is contained within an inverted repeat sequence that overlaps the transcription start site, and in the case of *amyL*, the translation initiation codon. A point mutation (*gra-10*) in this region of dyad symmetry relieves catabolite repression of *amyE* (Nicholson et al., 1987). In addition, Weickert and Chambliss (1989) have reported that deletion of DNA 3′ to

the *amyR1* promoter does not impair temporal activation of chloramphenicol acetyltransferase in *amyR1-cat86* transcriptional fusions, but abolishes catabolite repression.

Mutants of *B. subtilis* that are resistant to at least some manifestations of catabolite repression have been isolated. Among mutants originally selected for resistance to glucose-mediated repression of sporulation (Takahashi, 1979) is *crysA43*, which maps to rpoD, the structural gene for σ^A (Price and Doi, 1985). The alteration in σ^A in this variant also relieves catabolite repression in an *amyRi=lacZ* fusion cloned in *B. subtilis* (Laoide and McConnell, 1989). This may suggest a connection between the mechanism of glucose repression affecting sporulation and catabolite repression of at least those genes activated in stationary phase. In addition, Sun and Takahashi (1984) localized another catabolite-resistant mutation, *crysE1*, to the *rpo* operon. This locus encodes the β and β′ subunits of RNA polymerase. Taken together the two types of mutations occurring in subunits of RNA polymerase may suggest this enzyme is directly involved in catabolite repression or interacts with a protein, perhaps bound to the regulatory region, which mediates this phenomenon.

Resistance of Spores

The resting forms of bacteria are usually more resistant to various environmental stresses than their counterpart vegetative forms. The structure and composition of the resting (and dormant) form of endosporeforming bacteria, however, are quite different from other bacterial-resting forms (see Fig. 3). The core or protoplast containing the heat labile DNA, RNA, ribosomes, enzymes, and other proteins is surrounded by a primitive "germ cell wall." Moving toward the surface, there is a layer called the cortex which consists of peptidoglycan of a similar nature to that of the vegetative cell wall but with less cross linking in the peptides among other differences (Warth, 1978). A second cell membrane surrounds the cortex. The protoplast and cortex and their membranes are enclosed by layers of protein coat. A loose-fitting exosporium, appendages, and internal protein crystals may be found in some species.

Compared with vegetative cells, spores are more resistant to heat by a factor of 10^5 or more, to UV and ionizing radiation by 100-fold or more, and to desiccation, antibiotics, disinfectants, and other chemicals (reviewed by Roberts and Hitchins, 1969; Russell, 1982; Gould and Dring, 1974; Gould, 1983). Since spore resistance has been recognized for a long time and is important in food processing and sterilization

considerations, it has been extensively studied particularly with the genus *Bacillus*. Spore resistance to physical and chemical agents other than heat and irradiation indicated above is also noteworthy. Vegetative cells are killed at 88,000 p.s.i. for 14 h hydrostatic pressure while their spores have been shown to require 176,000 p.s.i. for the same time period (Gould and Dring, 1974). Spores are about 10,000 times more resistant to hyperchlorites than are vegetative cells. Two hundred roentgens (r) $\times 10^3$ of x-rays were required to kill 50% of treated *B. megaterium* spores, while 50% of treated *E. coli* cells were killed at 5.6×10^3 r. In every process designed for killing microorganisms, the spores are more resistant than vegetative cells and with few exceptions are the most resistant biological entities known. One exception is the nonsporeformer *Deinococcus radiodurans*, the most radiation-resistant organism yet discovered. Its resistance is presumably due to the possession of efficient repair mechanisms for radiation-induced changes in DNA.

The degree of heat resistance has been shown to depend not only on the species but also on the physiological environment in which the spores were formed. *B. stearothermophilus* spores are more heat resistant than *B. subtilis* spores which are more resistant than spores of *B. megaterium* (Roberts and Hitchins, 1969; Khoury et al., 1987). In addition, the spore resistance of each species depends on the temperature at which it was grown. For example, *B. subtilis* grown and sporulated at 20, 30, and 45°C produced spores having D_{90} values (the time required to kill 90% of the spores at 90°C) of 37, 78, and 99 min, respectively (Khoury et al., 1987). The spores were more temperature sensitive when formed in ethanol-supplemented media. Since temperature and ethanol are known to perturb the degree of order within membranes, this suggests that alteration of membrane function is an additional factor in the multifactorial nature of heat resistance. Other factors to consider in explaining heat resistance include protection of essential spore macromolecules (Murrell, 1981; Lindsay et al., 1985; Gerhardt and Marquis, 1989); specific effects of calcium dipicolinate (Lindsay and Murrell, 1986); mineralization (Marquis, 1989), and possibly foremost, dehydration (Gerhardt and Marquis, 1989). Current hypotheses on the heat resistance of endospores center on the dehydration of the protoplast (core) and the expandable cortex with its counterions (Gould, 1983). In the heat-resistant form, the spore coat is relatively impermeable to multivalent cations. The cortex (of high water content) contains an expanded electronegative peptidoglycan and mobile counterions exerting high osmotic pressure. The protoplast, of low water content, is osmotically dehydrated by the surrounding cortex and is, therefore, heat resistant. In the heat-sensitive form, there is a modified coat leaky to multivalent cations. The neutralized cortex, collapsed and free of counterions, exerts low osmotic pressure. The protoplast becomes partly hydrated and, therefore, heat sensitive. The theory of heat resistance, called the osmoregulatory expanded cortex theory, fits all the known facts but has yet to be proven or disproven. Germinated spores lose their heat resistance and yet, under special conditions, can be dehydrated to become both heat resistant and dormant once again (Gould, 1983). It is noteworthy that the heat resistance of some nonsporeformers can be increased in dehydrated cells. The expansion or contraction of the cortex is thought to account for the dehydrated state of the spore protoplast.

This reduced water content may also play a role in radiation resistance. It is thought that conformational differences between DNA in spore and vegetative cells may be associated with differences in hydration levels. The greater resistance of spores to UV radiation is also related to repair processes. The photoproduct formed during sporulation, 5-thyminyl-5,6-dehydrothymine (TDHT), is different from the thymine dimers formed in vegetative cells, both types of photoproducts being deemed as the cause of death. Dark repair mechanisms in germinating spores convert TDHT to thymine. The greater spore resistance to UV radiation is attributed to the more efficient removal of the unusual photoproduct (reviewed by Russell, 1982). However, as with the mechanism of spore heat resistance, the picture is complex and many factors are involved. For example, dipicolinic acid has been suggested as a protectant of spore DNA (Lindsay and Murrell, 1986) and it has been shown that the SASP may be involved (Setlow, 1988).

Likewise, explanations for ionizing radiation resistance of spores are not yet complete (Russell, 1982). Different spore DNA conformation (possibly due to dehydration), possession of coat layer radioprotectant substances, dipicolinic acid protection, and more efficient repair systems have all been implicated.

Spore coat layers, both coat protein and cortex, act as permeability barriers to toxic agents. At the present, this is the accepted explanation for the greater spore chemical resistance and resistance to lytic agents (Russell, 1982).

Spores as Biological Indicators

Endospores are often used as biological indicators. Known numbers of spores of various *Bacil-*

lus species of predictable death rate can be placed on various solid substrates (usually strips of filter paper) and placed with the items to be sterilized. The strips are then checked for retention of viable organisms by immersion into culture medium (various strategies are employed) after the sterilization process is completed if growth occurs then survival of the spores has occurred and hence the procedure for processing was inadequate. A D-value (decimal reduction time or time to kill 90% of the population at a particular temperature or treatment) of 3.0 min for *B. subtilis* spores is required for ethylene oxide sterilization (600 mg ethylene oxide per liter at 50% relative humidity and 54°C). For moist heat (121°C) *B. stearothermophilus* spores with a D-value of 1.5 min and for dry heat (170°C) spores of *B. subtilis* with a D-value of 0.8 min are preferred. *B. pumilus* spores (D-value of 0.17 Mrad) are used when ionizing radiation is used for sterilization (Korczynski, 1981).

Production of Antibiotics

Members of the genus *Bacillus* are capable of producing antibiotics as secondary metabolites in the late logarithmic or early stationary phase of growth of batch cultures. As many as 169 of these secondary metabolites have been recorded; for example, various strains of *B. subtilis* have been shown to produce 68 antibiotics while *B. brevis* can produce 23 (Katz and Demain, 1977). A partial updated listing modified from that of Katz and Demain (1977) is presented (Table 11) to illustrate that there are other *Bacillus* antibiotic producers. Most of the antibiotics are active against Gram-positive organisms, although there are exceptions. The majority are peptide antibiotics but some belong to other chemical classes (e.g., butirosin is an aminoglycoside and protocin is a phosphorus-containing triene). Also indicated in Table 11 are those antibiotics whose structural genes have been mapped and the few whose genes have been cloned. The latter types of studies are important in their own right and serve as models for the study of expression of the genes of other antibiotics.

A controversy has existed for some time concerning the function of these antibiotics. Since they usually appear upon the onset of sporulation, it has been proposed that they may be important factors in the transition of vegetative cells to spores (reviewed by Katz and Demain, 1977). Support for this hypothesis that

Table 11. Some *Bacillus* antibiotics.

Species	Antibiotic	Genes mapped	Genes cloned
B. subtilis	Subtilin[a]	+	+
	Surfactin[b]	+	−
	Bacilysin[a]	+	−
	Difficidin[c]	−	−
	Oxydifficidin[c]	−	−
	Bacillomycin F[d]	−	−
	Mycobacillin[d]	−	−
B. brevis	Gramicidin S[a]	+	+
	Lincar Gramicidin[a]	−	−
	Tyrocidin[a]	+	+
B. licheniformis	Bacitracin[a]	+	+
	Proticin	−	−
B. pumilus	Pumilin[a]	Remaining antibiotics have neither	
	Tetain	been mapped nor cloned.	
B. mesentericus	Esperin[a]		
B. polymyxa	Polymyxin[e]		
	Colistin[e]		
B. thiaminolyticus	Octopytin[a]		
	Baciphelacin[a]		
B. circulans	Circulin[e]		
	Butirosin[a]		
B. laterosporus	Laterospuramine[a]		
	Laterosporin[a]		
B. cereus	Biocerin[a]		
	Cerexin[a]		

[a]Anti-Gram-positive bacteria.
[b]Inhibitor of fibrin clotting.
[c]Broad spectrum antibiotic.
[d]Anti-fungal antibiotic.
[e]Anti-Gram-negative bacteria.

antibiotic production and sporulation may be regulated by the same or similar control mechanisms is now available. Transcription of the *B. subtilis tycA* gene (coding for tyrocidin synthesis) is dependent on the products of certain Stage 0 sporulation regulatory genes (Marahiel et al., 1987). Thus it does appear that the two physiological events are partially coupled to regulatory events occurring at the onset of sporulation.

In addition, it has recently been demonstrated that gramicidin S functions as an inhibitor of outgrowth after germination (Daher et al., 1985).

Protein Secretion

Members of the genus *Bacillus* are able to secrete a wide variety of enzymes into the culture medium (reviewed by Priest, 1977; Mezes and Lampen, 1985). Every *Bacillus* species which has been checked produces at least one extracellular enzyme. These include many different carbohydrates, several kinds of proteases, penicillinases, nucleases, phosphatases, lipase, phospholipase C, thiaminase, and bacteriolytic enzymes. A vast literature exists on the use of *Bacillus* enzyme models for studying secretion mechanisms, cellular location, and regulation. For some time, there has been considerable interest in producing large quantities of enzymes for industrial purposes—proteases for detergent supplementation, the brewing industry, various uses in the food industry and in leather manufacturing; and different amylases for brewery use, in bread making, and in the paper industry (see review by Debabov, 1982). Since many prokaryotic and eukaryotic genes can be fused to *B. subtilis*-derived regulatory regions and signal peptide sequences, such genetically manipulated organisms can be used for expressing and secreting many different heterologous proteins (for molecular cloning in *B. subtilis*, see Gryczan, 1982; Ganeson et al., 1982; Mezes and Lampen, 1985; Ganeson and Hoch, 1988). Interferon (Palva et al., 1983; Schien et al., 1986); human growth hormone (Honjo et al., 1986); and human interleukin-1 (Motley and Graham, 1988) are three such examples. However, yields of such proteins can be low, because extracellular neutral protease, subtilisin, esterases, and other proteases may degrade the secreted proteins. One strategy to overcome the problem and stabilize proteins has been to use strong glucose-insensitive promoters, protease deficient mutants, and catabolite repression of sporulation (Wong et al., 1986).

The widespread interest in protein secretion by *B. subtilis* has stimulated many studies on the genetics of secretion. There appears to be a general mechanism for regulating the synthesis of extracellular proteins. The activity of degradative enzymes can be increased by mutations at a number of loci that are unlinked to the structural genes for the affected enzymes. One family of genes, *senN*, *sacU*, *prtR* and *hpr*, codes for small regulatory proteins. For example, mutations at the *sacU* and *sacQ* loci can increase the expression of levan sucrase, alkaline protease, neutral protease, xylanase, betagluconase, alpha-amylase, and intracellular serine protease (Henner et al., 1988a). This type of stimulation of degradative enzymes is thought to be a global regulatory system turned on by a requirement for other carbon or nitrogen sources. The *SacU* product shares homology with other two-component sensorregulator systems (Ronson et al., 1987; Henner et al., 1988; Kunst et al., 1988).

Other Considerations

Just as *Bacillus* species have been important as models for studying differentiation or secretion, they have been employed extensively to study other important biological problems. These include DNA replication (Winston and Sueoka, 1982) and repair (Yasbin, 1985); chemotaxis (Ordal and Nettleton, 1985); genetic transformation (Dubnau, 1982); and translation apparatus (Smith, 1982; Hager and Rabinowitz, 1985).

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CHAPTER 1.2.17

The Genus *Bacillus—Insect Pathogens*

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Many different *Bacillus* species have been isolated from dead or dying insects. The *Bacillus* species commonly recognized as definite insect pathogens, however, are *B. popilliae*, *B. lentimorbus*, *B. larvae*, *B. thuringiensis*, and certain strains of *B. sphaericus* (de Barjac, 1981). These organisms, whose pathogenicity has been proved by fulfillment of Koch's postulates, are the subject of this review. The involvement of other *Bacillus* species in insect disease was reviewed by Krieg (1981) and several other authors in different chapters of the book *Microbial Control of Pests and Plant Diseases 1970–1980*, edited by H.D. Burges (1981).

Bacillus popilliae and Bacillus lentimorbus

B. popilliae and *B. lentimorbus* were named and described in 1940 by Dutky (1940). They are etiological agents of a naturally occurring disease of Japanese beetles, referred to as milky disease. The disease was so named because of the milky white appearance of the normally clear hemolymph of the diseased larvae (Hawley and White, 1935). The milkiness is due to the presence of very high numbers of spores of the bacteria; 2–5 $\times 10^9$ spores per larva or 2–5 $\times 10^{10}$ spores per ml (Dutky, 1940).

B. popilliae was named after the generic name of its host insect, the Japanese beetle, *Popillia japonica* Newman. The species *B. lentimorbus* was named by using the Latin adjective *lentus*, which means slow, and the Latin noun *morbus*, which means disease; literally, the slow disease (Dutky, 1940).

Both of these organisms have been used effectively for the biological control of the Japanese beetle (see Biotechnological Applications).

Habitats

Bacillus popilliae and *B. lentimorbus* are defined in nature as obligate pathogens according to the criteria of Bucher (1960). They are found only in association with a specific insect disease or in surrounding soil in the spore state. Both have a narrow host range, and in nature they probably grow only within the bodies of their host insects (Splittstoesser and Kawanishi, 1981). The host insects are all members of the order Coleoptera and the family Scarabaeidae.

Isolation, Growth, Maintenance, and Preservation of Cultures

These *Bacillus* species are isolated from hemolymph of infected beetle larvae where they occur as nearly pure cultures. Even when grubs of the Japanese beetle are co-infected with *B. popilliae* and *B. lentimorbus*, the diseased grubs prove to be infected with a single species, usually *B. popilliae* (Beard, 1946; Milner, 1981a).

To isolate, the grubs are first washed with water to remove most of the adhering soil, the surface is disinfected by immersion in 0.5% sodium hypochlorite (or 5% sodium hypochlorite [St. Julian et al., 1970]), the grub is rinsed, and the hemocoel is punctured with a dissecting needle. Hemolymph is then dripped into water. The spores can be freed of hemolymph by several alternating centrifugations and suspensions in water (Steinkraus, 1957a; Steinkraus and Tashiro, 1955).

Milner (1977) developed a useful, semiquantitative procedure for isolating a strain of *B. popilliae* (referred to as var. *rhopaea*) from soil. The procedure was based primarily on the observation that germination of *B. popilliae* spores was very slow in comparison to that of spores of other sporeformers. A soil suspension was made in a medium that promoted the germination of most spores except *B. popilliae*. The suspension was subjected to seven cycles of 40-min incubation followed by heating for 20 min at 70°C to kill germinated spores and vegetative cells. Further growth selectivity was provided by incubating the plated samples anaerobically (*B. popilliae* is facultative). Milner (1977) reported that about 15% of the *B. popilliae* spores in soil produced colonies and that a spore concentration of over 1.2×10^5 spores/g dry weight of soil could be quantified. Obviously, this procedure is very time consuming, and the possibility of quantitative error is great.

This chapter was taken unchanged from the second edition.

Both *B. popilliae* and *B. lentimorbus* are very fastidious. For example, neither will grow in nutrient broth, or if they grow, the growth is slight and ceases after three to four transfers following isolation from an insect host (Dutky, 1940). Spores or vegetative cells of both species can be grown on plates or in broth cultures of a variety of nutritionally rich, complex media. Two commonly used complex media are J-medium and MYPGP medium.

J-Medium for Growth of *B. popilliae* and *B. lentimorbus* (St. Julian et al., 1963)

Tryptone	5.0 g
Yeast extract	15.0 g
K_2HPO_4	3.0 g
Glucose (autoclaved separately)	2.0 g
Distilled water	to 1,000 ml

The pH is adjusted to 7.3–7.5. This medium may be solidified by inclusion of 20 g of agar.

MYPGP Medium for Growth of *B. popilliae* and *B. lentimorbus* (Costilow and Coulter, 1971)

Mueller-Hinton broth	10.0 g
Yeast extract	10.0 g
K_2HPO_4	3.0 g
Glucose (autoclaved separately)	0.5 g
Sodium pyruvate	1.0 g
Distilled water	to 1,000 ml

The pH is adjusted to 7.1. This medium may be solidified by inclusion of 20 g of agar.

Viable counts of vegetative cells or spores of *B. popilliae* and *B. lentimorbus* can be made by direct plating of suitable dilutions onto either of these media. The diluent should be either growth medium or 0.1% tryptone. Dilution in water or 0.85% NaCl leads to a rapid decline in viability (St. Julian et al., 1963). (If vegetative cells or spores are used to inject into grubs, the tryptone diluent is recommended because of the toxicity of growth medium [St. Julian et al., 1963].)

Inefficient germination and/or outgrowth complicates determination of spore numbers. It is common for 1 to 5% of microscopically visible spores to produce colonies. Aging of spores at 1–5°C (Splittstoesser and Farkas, 1966; Tashiro, 1957) or at room temperature (Milner, 1981a) provides some improvement. The other commonly used procedure is activation of germination by heating at 50°C for 15 min, although the effect is not dramatic. Sharpe et al. (1970) reported that 3.3% of microscopically visible spores of *B. popilliae* NRRL B-2309M produced colonies compared to 5.7% after 15 min of heating at 50°C. Heating at 60 to 80°C resulted in fewer colonies (St. Julian and Hall, 1968). St. Julian et al. (1967) suspended spores of *B. popilliae* in various solutions prior to heating at 50°C for 20 min and plating. Adenosine, L-alanine, and glucose, alone and in combination, had no effect on the plating efficiency. Also, KCl, NaCl,

$MnCl_2$, $CaCl_2$, $MgCl_2$, K_2HPO_4, and Na_2HPO_4 had little or no effect. Treatment of the spores with lysozyme, trypsin, and crude snail enzyme (from *Helix pomatia*) alone and in combination with various sugars had no effect. Splittstoesser and Farkas (1966) and Splittstoesser and Steinkraus (1962) reported that heating spores of *B. popilliae* at 60°C for 15 min in $CaCl_2$ (1 mM) at pH 7 caused about a 1,000-fold increase in colony count. This effect was prevented by KCl in concentrations as low as 10^{-6} M. There is no clear reason for the discrepancies between the results of the two groups with regard to heating in the presence of $CaCl_2$ and KCl. Perhaps the age of the spores was different. Splittstoesser and Farkas (1966) used spores freshly harvested from infected grubs. The age of the spores used by St. Julian et al. (1967) was not specified. Costilow and Coulter (1971) reported that spores of *B. popilliae* NRRL B-2309M heat shocked at 60°C for 15 min exhibited a plating efficiency of 60% when plated on the MYPGP medium as described above. Pyruvate was found to be important; when it was excluded from the medium, 7.7% plating efficiency occurred. Hrubant and Rhodes (1969) reported that treatment of spores of *B. popilliae* NRRL B-2309S with a crude extracellular enzyme produced by an unidentified *Bacillus* species (NRRL B-3425) increased the percentage germination from 8–16% to 36–43%. Splittstoesser et al. (1975) observed germination and outgrowth microscopically. They reported that more than 90% of spores of *B. popilliae* germinated and outgrew within 1 h when they were suspended in cabbage looper hemolymph and tyrosinase (quantity not specified), at an alkaline pH (unspecified) at 37°C.

Cells of *B. popilliae* and *B. lentimorbus* can be maintained in the vegetative state by transferring cultures from J-medium or MYPGP agar to fresh medium once every 7 to 10 days. The plates or tubes should be incubated at 30–33°C until pinpoint colonies are present, and then refrigerated. Gordon et al. (1973) employed monthly transfers of *B. popilliae* in tubes of J-medium without glucose and to which 0.1% agar was added and incubated at 25°C for the entire month. For *B. lentimorbus*, stock cultures were maintained in a biphasic medium (Haynes and Rhodes, 1963) on a shaker at 25°C. Equal volumes of $2 \times$ J-broth without glucose and 4% agar were sterilized separately. The agar was allowed to solidify and the broth was then added. Inoculum (5%) was transferred every 2 weeks.

The most convenient way to maintain cultures for long periods of time is to make use of the natural longevity of spores. Washed spore suspensions can be added to dry, sterile soil, smeared on glass microscope slides and dried,

or kept as suspensions (in water or alcohol) (Milner, 1981a; St. Julian et al., 1978). Another way of maintaining cultures is to spread hemolymph from infected grubs directly (without washing the spores) onto slides and to air-dry them (Milner, 1981a). The problem with these approaches is that spores are formed reproducibly only by infection of their host larvae (see "Ecophysiology; Growth and Sporulation In Vitro," this chapter).

Lyophilization has been used to preserve vegetative cells of *B. popilliae* and *B. lentimorbus*. Haynes et al. (1961) scraped cells of both species from agar-solidified medium and suspended them in bovine serum prior to lyophilization. Lingg et al. (1967) prepared cells for lyophilization by suspending them in a solution containing 5% sodium glutamate and 0.5% gum tragacanth.

Identification

No phylogenetic studies have been conducted on *B. popilliae* and *B. lentimorbus*. From the taxonomic studies that have been conducted, mainly based on phenotypic observations, these species do belong in the genus *Bacillus* (Claus and Berkely, 1986). They are rod-shaped, endospore-formers (with swollen sporangia) that have a typical Gram-positive cell wall structure (Black, 1968a, 1968b), the latter as observed by electron microscopy. (Actually, the Gram-staining reaction is reported to be negative except during the sporulation process.) They are facultative organisms, although they grow much better aerobically (Sharpe, 1966). One characteristic that makes these two species (and *B. larvae*) unusual members of the genus *Bacillus* is that they lack catalase. All other members of the genus, except *B. azotoformans* and some strains of *B. stearothermophilus*, are catalasepositive (Claus and Berkeley, 1986).

B. popilliae and *B. lentimorbus* are very similar, both causing milky disease in a restricted group of beetles belonging to the family Scarabaeidae. Of 42 phenotypic characteristics recommended by Claus and Berkeley (1986) for differentiation of *Bacillus* species, these two species share all but two; only *B. popilliae* produces a parasporal crystalline inclusion and is capable of growth in 2% NaCl. [The latter characteristic may be strain variable; Milner (1974) reported that a strain he refers to as *B. popilliae* var. *rhopaea* has a parasporal body but is incapable of growth in 2% NaCl.] Kaneda (1977) divided 19 *Bacillus* species into six groups based on fatty acid composition. *B. popilliae* and *B. lentimorbus* were placed in group B with *B. larvae* and *B. polymyxa* (see The Genus *Bacillas—Nonmedical* in this Volume). This group's fatty acids consisted of less than 3% unsaturated fatty acids. The predominant (39–62%) fatty acid was the anteiso-C_{15} acid (range of chain length; 14–17) (Kaneda, 1969, 1977).

Dutky (1940) noted that there was a noticeable difference in the diseases caused by *B. popilliae* and *B. lentimorbus*. *B. popilliae* caused "type A milky disease," in which the infected grubs became increasingly white and opaque during the course of the infection. During the last stage of the disease, just before death, the grubs exhibited a slight brownish tinge. *B. lentimorbus* caused "type B milky disease." This disease state was superficially the same as type A in infected grubs found in late summer and fall. In grubs infected in the fall that survived until spring, however, a marked difference was noted, the color of these grubs turned a muddy brown. This darkening was due to extensive formation of blood clots, which are brown to black. Clots that accumulated in appendages blocked the insects' circulation, leading to a blackened, gangrenous condition of the affected parts. The biochemical basis of this clotting phenomenon is unknown.

Other differences between *B. popilliae* and *B. lentimorbus* have been reported. They differ in lipid composition (Bulla et al., 1970a; Kaneda, 1969), and they differ somewhat in the surface topography of their ridged spores (Bulla et al., 1969). Hrubant and Rhodes (1968) showed that antisera in rabbits presumably against surface antigens of B. popilliae and *B. lentimorbus* (the cells were incubated overnight at 4°C in 0.85% NaCl and 0.5% phenol before use as antigens). Antiserum against *B. popilliae* showed no cross-reaction in agglutination tests against *B. lentimorbus* cells and vice versa. Conversely, antisera made in rabbits against sonicated vegetative cells of both species showed significant cross-reaction in double diffusion assays and immunoelectrophoresis (Krywienczyk and Luthy, 1974).

Other data that are sometimes of taxonomic value relate to the type of peptidoglycan (Schleifer and Kandler, 1972) and the major type of quinone (Collins and Jones, 1981) possessed by cultures. Comparative data on *B. popilliae* and *B. lentimorbus*, however, are lacking. The *B. lentimorbus* peptidoglycan is of the direct cross-linked *meso*-diaminopimelate type [from Ranftl (1972) as cited by Schleifer and Kandler (1972)]. No information exists on the peptidoglycan type of *B. popilliae*. The major quinone possessed by *B. popilliae* is a menaquinone with seven isoprene units (MK-7), but no information exists on the quinone(s) possessed by *B. lentimorbus* (Hess et al., 1979).

From the above and other data, it is understandable that differences of opinion have arisen concerning the taxonomy of these bacteria. Claus and Berkeley (1986), Gordon et al. (1973), and Krieg (1961) have preferred to consider

these two as separate species, giving weight to the presence or absence of the parasporal body. Krywienczyk and Luthy (1974), Milner (1981a), and Wyss (1971) have given more weight to characteristics other than the parasporal body, and, thus, consider it more appropriate to refer to "*B. lentimorbus*" as *B. popilliae* var. *lentimorbus* and "*B. popilliae*" as *B. popilliae* var. *popilliae*.

Species names other than *B. popilliae* and *B. lentimorbus* have been proposed for some milky disease isolates. A bacterium was isolated in 1956 by Beard from an Australian Scarabaeidae, specifically *Heteronychus sanctae-helenae* Blanch, that had a milky disease. A very similar strain, referred to as *B. lentimorbus* var. Maryland, was studied by Tashiro and Steinkraus (1966) and Steinkraus and Tashiro (1967). Beard (1956) believed that his isolate differed sufficiently in morphology and in host specificity from *B. lentimorbus* that it deserved separate species status; he called it *B. eulomarahae*. The sizes of the vegetative cell rods and the spores of both Beard's isolate and *B. lentimorbus* var. Maryland are 0.3 × 3 µm and 0.2–0.4 µm in diameter, respectively. This compares to the corresponding dimensions of *B. lentimorbus* (Dutky, 1940) of 1×5 µm and 0.9×1.8 µm, respectively (Beard, 1956). These are rather significant differences. Studies on many more strains, including use of molecular taxonomic techniques, are required before this species designation should be officially recognized.

A separate species designation was also given to a culture isolated from a milky diseased common cockchafer grub (*Melolontha melolontha* Linneaus) in Switzerland by Wille (1956); i.e., *Bacillus fribourgensis*. Hurpin (1966) isolated another culture from a diseased grub of *M. melolontha* in France, and designated it as *B. popilliae* var. *melolontha*. These two cultures were reported to be essentially identical in appearance of the parasporal body, in their ability to grow and sporulate in tissue cultures of *Phyllophaga anxia* hemocytes (Lüthy et al., 1970), in their antigenic composition based on double diffusion and complement fixation (Lüthy and Krywienczyk, 1972), and in their nutritional requirements (Wyss, 1971). They differed from the original *Bacillus popilliae* isolate of Dutky (1940) in host specificity, virulence for *M. melolontha* L. by different routes (Hurpin, 1967), nutritional requirements (Wyss, 1971), and a lower maximal growth temperature (33°C as compared to 37°C for *B. popilliae* Dutky) (Wyss, 1971). Most investigators agree that separate varietal status is probably more appropriate than separate species status.

Milner (1974) in Australia isolated a parasporal body-containing strain of *B. popilliae* from *Rhopaea verreauxi* that differed from both the Dutky (1940) strain (which he referred to as *B. popilliae* var. *popilliae*) and *B. popilliae* var.

melolontha. It differed in host specificity (Milner, 1974, 1976), maximum growth temperature (32°C) (Milner, 1974), the ability to use carbohydrates and salicylic acid (it cannot use fructose and salicylic acid, compounds used by the other two varieties) (Milner, 1974; Wyss, 1971), and the inability to grow in 2% NaCl (Milner, 1974).

Other strains of milky disease-causing bacteria exist that possess parasporal bodies that should be investigated for possible varietal status in the species *B. popilliae*. Dumbleton (1945) in New Zealand isolated a culture from *Odontria zealandica* White that differs from *B. popilliae* var. *popilliae* in the shape of the parasporal body and in host specificity. White (1947) and later Adams (1949), Harris (1959), and Dutky (1963) studied milky disease in *Cyclocephala borealis*, the northern masked chafer. The *Bacillus popilliae* strain isolated from this species is unusual in host specificity and in having large and sometimes multiple parasporal bodies. Recently Boucias et al. (1986) found that milky disease of a serious sugarcane pest in Florida, *Cyclocephala parallela* Casey, was caused by *B. popilliae*. Fowler (1972, 1974) isolated a *B. popilliae* strain from the New Zealand grass grub *Costelytra zealandica* and White (1986) in India isolated a strain of *B. popilliae* referred to by Milner as *B. popilliae* var. *holotrichia* from *Heterodera* (= *Holotrichia*) *consanguinea* (Blanchard), a grub that causes damage to sugarcane and other wet-season crops. Feng et al. (1982) in China studied a strain of *B. popilliae* designated SH-m5 that has pathogenicity for *Holotrichia oblita* Fald., *Anomala corpulenta* Mots., and *Exolontha serrulata* (Gyllenhal).

Probably different varieties also exist in the species *Bacillus lentimorbus*. For example, Beard (1956) in Australia referred to a culture that he isolated from milky diseased *Sericesthis pruinosa* (Dalm.) as *B. lentimorbus* var. *australis*. It differed from the originally isolated *B. lentimorbus* (Dutky, 1940) in host specificity. Other strains that should be studied include a strain lacking a parasporal body that was isolated from the New Zealand grass grub *Costelytra zealandica* (Fowler, 1972, 1974), strain RM17 isolated in Australia from *Aphodius tasmaniae* (Milner, 1981b; Milner and Beaton, 1981), and the strains isolated by Boucias (1986) in Florida from *Ligyrus subtropicus* Blatchley, the worst pest of Florida sugarcane. Investigations on taxonomy of *Bacillus lentimorbus* have definitely been hampered by the difficulty in growing the organism. Some strains, for example RM17 mentioned above (Milner, 1981b), have not yet been cultivated on artificial media.

In conclusion, considerable confusion exists concerning the appropriate nomenclature for milky disease bacteria. It is not clear whether *B. popilliae* and *B. lentimorbus* should be consid-

ered separate species. Varietal designations may or may not be appropriate. Phage typing would aid in these considerations but no phages active on these organisms have been discovered. Immunological techniques have only been used to a small extent. Molecular taxonomic approaches would be of great value with regard to these issues. For example, De Ley (1978) states that to belong to a single species, cultures should have GC contents that vary by no more than 2%. The reported GC content of *B. lentimorbus* and *B. popilliae* are 37.7 and 41.3mol%, respectively (reference is not an exact match-Manichini et al., 1968), thus suggesting that separate species designation is appropriate. This conclusion is rendered less certain by the report of Fahmy et al. (1985) that the GC values for the same strain of some *Bacillus* species published by different investigators varied widely (up to 14%). Nucleic acid hybridization would be of even greater value than DNA base composition, but no data exist for *B. popilliae* and *B. lentimorbus*. It is perhaps appropriate to maintain separate species status, as has been done by the authors of the section on *Bacillus* species in the current edition of *Bergey's Manual of Systematic Bacteriology* (Claus and Berkeley, 1986), until more comparative data have been obtained.

Ecophysiology

NUTRITION. Both *B. popilliae* and *B. lentimorbus* are very fastidious. Most of the media in common use employ yeast extract, casein digest, and a carbohydrate, the latter reported to be essential for growth as an energy source (Bulla et al., 1978; Rhodes et al., 1966). Defined synthetic media have been developed (Sylvester and Costilow, 1964; Wyss, 1971). Thiamine is essential for growth, a discovery first made by Dutky (1947, 1963). Biotin, although not essential, is stimulatory for growth. Eleven amino acids are essential and three are stimulatory. Purines and pyrimidines are not required. Sylvester and Costilow (1964) reported that inclusion of barbituric acid is required for growth in their medium. The role of barbituric acid is unknown (Coulter and Costilow, 1970; Sylvester and Costilow, 1964).

Carbohydrates that can be used by all varieties of *B. popilliae* and *B. lentimorbus* tested include glucose, galactose, mannose, maltose, and trehalose (Milner, 1974; Steinkraus and Tashiro, 1967). All varieties tested except for *B. popilliae* var. *rhopaea* use fructose (Milner, 1974). No hydrolysis of starch, casein, or gelatin occurs (Claus and Berkeley, 1986).

These bacteria are facultative, but their growth is greatly enhanced by the presence of oxygen, as will be discussed below (see "Metabolism" this chapter).

GROWTH AND SPORULATION IN VIVO. The normal route of infection of these microbes is by feeding. St. Julian et al. (1970) described the infectious process as occurring in four phases: 1) during the first two days no bacteria are present in the hemolymph; 2) vegetative proliferation predominates from day 3 to day 5; 3) from day 5 to day 10 vegetative growth and sporulation occur concomitantly; and 4) the last phase, which usually terminates with insect death by day 14 to 21, is characterized by massive sporulation. As indicated previously, the number of spores at the time of death is as high as 5×10^{10}/ml. The insect grub continues feeding and appears normal until near death.

The most detailed description of how the bacteria migrate from the grub's intestinal tract into the hemolymph deals with infection by *Bacillus popilliae* var. *popilliae* of the European chafer, *Amphimallon majalis* (Splittstoesser et al., 1973; Splittstoesser et al., 1978). The spores germinate in the hindgut and vegetative cells are transported to the midgut by antiperistalsis. Here the vegetative cells penetrate the epithelial cells lining the midgut, mostly at the anterior end. This penetration seems to be by a phagocytosis-like event. Nothing is known about the mechanism of attachment that must precede phagocytosis. Perhaps the glucose- and mannose-containing capsular polysaccharide (Li et al., 1985) is involved. At the area of intracellular intrusion, hemocytes aggregate to form an inflammatory capsule on the hemocoel surface of the digestive tract. Considerable bacterial cell death occurs during this intracellular migration but a few viable cells pass into the hemolymph where they proliferate and sporulate. Once in the hemolymph, no phagocytosis by hemocytes occurs. Splittstoesser et al. (1973) indicated that bacilli tend to concentrate in connective tissue sheaths or in close contact with hemocytes during the sporulation process. Schwartz and Townshend (1968) reported that *B. popilliae* spores and vegetative cells in hemolymph of Japanese beetle larvae are also not phagocytized. They noticed no effect of the bacteria on hemolymph coagulation or on the number of hemocytes.

In the case of *B. thuringiensis*, the parasporal body alone causes most of the disease symptoms and may also aid in bacterial penetration to the hemocoel. With milky disease caused by *B. popilliae*, there is no such clear evidence. *B. lentimorbus* causes a disease that is almost identical to that caused by *B. popilliae*, and has no parasporal inclusion. Weiner (1978) isolated parasporal bodies from *B. popilliae* and determined that they were proteinaceous in nature. Whole or alkali-solubilized parasporal bodies fed to third-instar larvae of Japanese beetles were not toxic. Injection of solubilized parasporal bodies did cause death. Solubilized parasporal protein of

B. popilliae var. *melolonthae* also causes loss of viability of *Melolontha melolontha* primary hemocyte cultures (Lüthy et al., 1976).

The precise cause of death is also not understood. Dutky (1963) observed that thiamine present in the hemolymph of healthy Japanese beetle larvae was absent in hemolymph of milky-diseased larvae. This observation led him to speculate that larval death was due to starvation for essential nutrients. Lüthy (1986) speculated that death is simply due to general exhaustion coured by the heavy infection. There is a possibility that one or more toxins may be involved since cell-free filtrates of cultures of *B. popilliae* are lethal upon injection (Dutky, 1963). The toxic component(s) is inactivated by heating at 50°C for 10 min. It is unknown whether this toxin(s) is elaborated in vivo. Sharpe and Detroy (1979) found that the fat bodies of Japanese beetle larvae infected with *B. popilliae* were reduced in weight about 75% from those of healthy larvae. They suggest that this depletion may cause death simply by preventing progress to the pupal stage.

One approach used to gain an understanding of conditions promoting growth and sporulation in the larval hemolymph of Japanese beetles was to determine changes that occurred in the hemolymph during the development of milky disease caused by *B. popilliae*. The results from a decade of research, including references to many publications, were published in a review by Bennett and Shotwell (1973), and will not be discussed in this review, except as they relate to the subsequent discussion of metabolism (see "Metabolism," this chapter).

Growth and Sporulation In Vitro. Growth of *Bacillus popilliae* is exponential in most complex media used. After the maximum population is attained, there is usually a very rapid decline in cell viability with little or no sporulation occurring (Rhodes et al., 1966). The lack of efficient sporulation in vitro has been the major factor limiting the usefulness of these bacteria for insect control (see "Biotechnological Applications" this chapter). Only spores can be used for biological control, because of the rapid loss of viability in soil that occurs with vegetative cells.

Steinkraus and Tashiro (1955) were the first to obtain sporulation of *B. popilliae* and *B. lentimorbus* in vitro. Their strategy was based on the hypothesis that sporulation was induced by nutrient deficiency. They transferred cells growing on a complete solid medium to the surface of an agarsolidified starvation medium as a paste. Some sporulation occurred, but the spores fed to larvae were reduced in virulence. In a separate study, no significant sporulation of *B. popilliae* occurred in European chafer hemolymph, mac-erated larvae, or larval extracts (Steinkraus, 1957b). (Efficient sporulation occurs in the hemolymph of viable larvae of this insect host.)

Rhodes et al. (1965) obtained up to 0.3% sporulation of *B. popilliae* by the use of a solid medium that contained yeast extract, sodium acetate, potassium phosphate, and agar. The strain used was a derivative of strain NRRL B-2309, referred to as NRRL B-2309S, because it was isolated from a smooth colony on acetate agar. Addition of glucose or trehalose repressed sporulation. Sporulation frequency was unchanged by addition of soil extract, Japanese beetle hemolymph, or larval extract. It was later found that they could occasionally obtain between 10^5 and 10^7 spores/ml of this strain in liquid J-medium supplemented with 1.0% charcoal (Haynes and Rhodes, 1966; Haynes and Rhodes, 1969). The brand and specific batch of charcoal (Haynes et al., 1972), yeast extract (Haynes and Crowell, 1973), and tryptone (Haynes and Crowell, 1973) were crucial. Haynes and Whih (1972) reported that it was important to use spores from the previous culture of strain B-2309S as inoculum. Use of vegetative inocula resulted in loss of ability to sporulate. It is unknown whether spores of B-2309S are infective by feeding; they are by injection (St. Julian and Bulla, 1973).

Sharpe et al. (1970) isolated another strain of *B. popilliae*, referred to as NRRL B-2309M, from strain B-2309S. They developed an MYPT medium containing Mueller-Hinton broth, yeast extract, potassium phosphate, and trehalose that supported fair sporulation (up to 1.5×10^6 spores/ml). Sporulation was improved when the medium was filter-sterilized, and best when on solid MYPT medium (Sharpe et al., 1970; Sharpe and Rhodes, 1973). Asporogenic sectors frequently appeared in colonies. Although strain B-2309M sporulates fairly well in vitro, the spores are not commercially useful because they are infective for Japanese beetle larvae only by injection, not by feeding (Schwartz and Sharpe, 1970; Sharpe et al., 1970).

Costilow and Coulter (1971) developed MYPGP agar medium (see "Isolation, Growth, Maintenance, and Preservation of Cultures," this chapter), that supported 12% sporulation of *B. popilliae* NRRL B-2309M. The pyruvate in the medium was shown to be essential for sporulation.

Costilow et al. developed a broth medium and cultural conditions that resulted in the production of refractile bodies in cells of *B. popilliae* and *B. lentimorbus* that were not mature spores but could be premature, abortive spores (Costilow et al., 1966; Mitruka et al., 1967). Spores and the refractile bodies ("Costilow Bodies") of *B. popilliae* were similar in RNA, DNA,

and protein content. Refractile bodies did not contain dipicolinic acid, whereas spores contained 1.9% dipicolinate. Catalase, although absent from vegetative cells, was present at low levels in both spores and refractile bodies (Mitruka et al., 1967).

Yousten et al. (1974) attempted, without success, to obtain sporulation of *B. popilliae* NRRL B-2309 by resuspension of postexponential phase cells of *B. popilliae* in glucose-supplemented spent broth prepared from a sporogenous strain of *B. subtilis*. Although unsuccessful, this approach was reasonable, particularly since the recent discovery of the extracellular factor(s) produced by *B. subtilis* that is required for efficient sporulation (Grossman and Losick, 1988).

Sporulation up to 50% was reported to occur when *B. popilliae* var. *melelontha* was grown in tissue culture medium with hemocytes of *Phyllophaga anxia* larvae (a June beetle) (Lüthy et al., 1970) or hemocytes of *Melolontha melolontha* larvae (Lüthy et al., 1976). The spores were infective by feeding. *Bacillus popilliae* NRRL B-2309 (var. *popilliae*) grew but did not sporulate in either of these systems. Although the above results are promising, tissue culture techniques are too laborious and expensive to be useful for mass production of spores (Lüthy et al., 1976).

Results recently reported by Feng et al. (1982) are promising. They claim to have observed 50–60% sporulation when *B. popilliae* SH-m5 was cultured in J-broth supplemented with thiamine-HC1 (0.01 g/l). Moreover, the spores produced in vitro were infective by feeding as well as by injection into larvae of three different species: *Holotrichia oblita* Fald., *Anomala corpulenta* Mots., and *Exolontha serrulata* (Gyllenhal).

METABOLISM. *Bacillus popilliae* and *B. lentimorbus* are facultative microbes but their growth is definitely enhanced in the presence of oxygen. Sharpe (1966) showed that the growth of *B. popilliae* in a fermenter was optimal at an aeration rate of 0.5 vol/vol/min (vvm), an air-flow equivalent to an oxygen absorption rate of 0.5 mmol of oxygen/l/min. Weiner (see Rhodes, 1965) measured the dissolved oxygen concentration during growth of *B. popilliae* in a fermenter. As growth proceeded, a large drop in dissolved oxygen occurred. When growth was complete (and cell viability decreased) the dissolved oxygen concentration increased to a normal value. The hemolymph of healthy Japanese beetle third-instar larvae has a dissolved oxygen concentration of 0.078–0.1 μmol/ml (Weiner et al., 1966). When vegetative cells are abundant, a 36–53% drop in dissolved oxygen occurs, followed by a return to a normal level as sporulation proceeds (Weiner et al., 1966).

There are some indications in the literature that sporulation of *B. popilliae* is somewhat oxygen sensitive. The rapid loss in viability commonly observed (Rhodes et al., 1966) when growth of *B. popilliae* in liquid media is complete obviously precludes efficient sporulation. Costilow et al. (1966) reported that cessation of shaking at the end of the growth phase decreased the death rate. Sharpe et al. (1970) obtained optimal sporulation of *B. popilliae* NRRL B-2309S by incubating cells in MYPT broth with shaking for 3 days followed by no shaking for 10 to 15 days. The fact that sporulation occurs best on solid rather than in broth media may also be due to the reduced oxygen microenvironment, which is undoubtedly present when oxygen-metabolizing cells are so closely packed together. Oxygen is required for sporulation (Sharpe et al., 1970; Sharpe and Rhodes, 1973), but there may be a critical stage that requires reduced oxygen concentration.

B. popilliae, in addition to being catalase-negative as determined by the loop test, also lacks NADH peroxidase (Pepper and Costilow, 1965). Costilow and Coulter (1971) did detect some catalase in spores, but the activity was thousands-fold less than that in more common *Bacillus* species such as *B. subtilis* (Dingman and Stahly, 1984). Pepper and Costilow (1965) and Costilow et al. (1966) hypothesized that in *B. popilliae* the production of H_2O_2 during the stationary phase may be responsible for the rapid loss of viability and consequent absence of sporulation. Cell extracts of late exponential or early stationary phase cells produce H_2O_2 via a soluble NADH oxidation system (Pepper and Costilow, 1965). Attempts to prevent loss of viability by addition of catalase to cultures have been unsuccessful (Costilow et al., 1966; Steinkraus, 1957b). However, this finding does not rule out the possibility that intracellular H_2O_2 accumulation is responsible for the loss of viability. In larval hemolymph, the oxygen concentration may be poised perfectly so that the amount of available oxygen is adequate to provide the energy needed for sporulation but not enough to favor H_2O_2 accumulation. One other enzyme required for protection against oxygen toxicity is superoxide dismutase, the enzyme that catalyzes the conversion of superoxide (O_2-) to H_2O_2 and O_2. Superoxide dismutase is produced by *B. popilliae* at an activity level approximately equivalent to that of *Escherichia coli* (Costilow and Keele, 1972). Only a single form of superoxide dismutase is synthesized in *B. popilliae* (Yousten and Nelson, 1976) and it is induced by aerobic growth (Yousten et al., 1973).

Both *B. popilliae* and *B. lentimorbus* oxidize glucose, primarily to acetate and lactate. Small amounts of glycerol and ethanol and traces of

acetoin and acetaldehyde accumulate (Pepper and Costilow, 1964). The ratio of acetate to lactate produced by *B. popilliae* increases with increased aeration. Cells incubated anaerobically do not catabolize glucose. Glucose is catabolized by both *Bacillus* species solely by the Embden-Myerhof-Parnas (EMP) and the pentose phosphate (PP) pathways (Bulla et al., 1970b; Pepper and Costilow, 1964). Under normal aeration, glucose is catabolized by *B. popilliae* primarily (75–98%) by the EMP pathway (Bulla et al., 1970b; Pepper and Costilow, 1964). When the cells are aerated with 100% oxygen, 60% of the glucose is oxidized via the EMP pathway and 40% by the PP pathway (Pepper and Costilow, 1964). When *B. popilliae* cells were harvested from hemolymph of infected Japanese beetle larvae during growth and sporulation, they catabolized 75% of the glucose by the PP pathway and 25% by the EMP pathway; a pattern opposite from that existing for cells grown in vitro (St. Julian et al., 1975). It is possible that the oxygen concentration in vivo is the factor that favors the PP pathway, but one or more different control mechanisms may be operative.

In larvae, glucose is not the major carbohydrate used by these bacteria. Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is the major carbohydrate in Japanese beetle hemolymph. It is present at very high concentrations, about 0.7%, a concentration that inhibits vegetative growth of *B. popilliae* in vitro (Bennett and Shotwell, 1973). This fact led Bennett and Shotwell (1973) to speculate that trehalose might be present in hemolymph in a bound form to be released as needed by the pathogen. During sporulation in vivo, the concentration of trehalose is reduced to between 0.1 and 0.47% (Bennett and Shotwell, 1973). Thus, there is a significant concentration of trehalose present during sporulation. Although in vitro sporulation of *B. popilliae* NRRL B-2309S is repressed by trehalose (Rhodes et al., 1965), it is possible that trehalose is actually required for in vivo sporulation. Trehalose is transported into cells by a phosphotransferase system using phosphoenolpyruvate (PEP) as the phosphoryl donor (Bhumiratana et al., 1974). [The uptake mechanism used for glucose transport is unknown. No PEP-glucose phosphotransferase has been detected (St. Julian et al., 1975).] The intracellular transported form of trehalose, 6–0-phosphoryl-α-D-glucopyranosyl-α-D-glucopyranoside, is cleaved to glucose and glucose 6-phosphate by phosphotrehalase, a reaction first detected in *B. popilliae* (Bhumiratana et al., 1974). The intracellular level of PEP may regulate the uptake of trehalose. PEP is more likely to accumulate when the EMP pathway is used rather than the PP pathway. Thus, Bulla et al. (1978) proposed that perhaps the dependence on the EMP pathway by in vitro grown cells results in excessive accumulation of PEP, which, in turn, results in excessive intracellular accumulation of trehalose that represses sporulation.

Most *Bacillus* species oxidize glucose to pyruvate, lactate, or acetate during growth. When the glucose is gone, growth ceases, synthesis of enzymes of the tricarboxylic acid (TCA) cycle is induced, and the acids that accumulated during growth are oxidized to CO_2 and H_2O (Hanson et al., 1963, 1964; Nakata and Halvorson, 1960). *B. lentimorbus* NRRL B-2522 and *B. popilliae* B-2309 cells grown in vitro or in vivo (*B. popilliae* only) were not capable of oxidizing acetate (Bulla et al., 1971; McKay et al., 1971; St. Julian et al., 1975). Pepper and Costilow (1964) reported that a variant of strain B-2309, strain B-2309 PA, was able to oxidize acetate. This oxidation occurred at the end of the exponential growth phase. McKay et al. (1971) showed that several other variants of strain B-2309 also were able to oxidize acetate and they presented evidence in support of the existence of a complete TCA cycle. Although some of these strains are oligosporogenic in vitro, the strains that oxidize acetate most rapidly do not sporulate in vitro (McKay et al., 1971). It is possible that operation of a TCA cycle is not required for sporulation in vivo. The constant supply of trehalose during sporulation may serve as an adequate energy source (Bulla et al., 1978).

Genetics

A problem that complicates scientific study and commercial use of these organisms is the presence of genetic instability. The loss in ability to sporulate after prolonged cultivation on artificial media is a commonly observed phenomenon. Sharpe and Bulla (1978) reported that *Bacillus popilliae* NRRL B-2309 cells when plated yielded three different colonial variants, each of which exhibited a different generation time. Each colony type reverted to a mixture of all three types, but the tendency was to transform to the fastest growing variant, which proved to be noninfective when injected into Japanese beetle larvae. There seemed to be a correlation between slow growth rate and virulence.

Practically nothing is known about the genetics of these organisms. No conjugation or transduction system is known. In fact, no phages have been discovered that infect these bacteria. Bakhiet and Stahly (1986) developed a protoplast transformation system that was effective in permitting the transformation of plasmid pHV33 into *B. popilliae* NRRL B-2309S, but the frequency of transformation was so low that transformation attempts were often unsuccessful.

One finding that facilitated production of protoplasts was that cell walls of *B. popilliae* are degraded by mutanolysin; they are resistant to lysozyme (Bakhiet and Stahly, 1985a). Development of a more efficient method of transformation will be a significant step toward gaining an understanding of the genetics of these organisms.

The only published information on plasmids present in these species is the report of Faust et al. (1979) that there are two plasmids present in the strain of *B. popilliae* isolated from spore powder produced by Fairfax Biological Laboratory, Inc., Clinton Corners, New York.

D. W. Dingman (personal communication) found that chromosomal DNA of *B. popilliae* (nine strains) and *B. lentimorbus* (one strain) is resistant and sensitive to restriction by *Mbo*I and *Dpn*I, respectively. Chromosomal DNA of each of seven other *Bacillus* species is restricted by *Mbo*I but not *Dpn*I. This is evidence for the presence of ^{6}N-methyl adenine in the GATC sequences in DNA of *B. popilliae* and *B. lentimorbus*. Modification of this sequence is presumed to occur by a DNA methylase which functions like the *dam* methylase of *E. coli* (Marinus and Morris, 1973; Lacks and Greenberg, 1977).

Biotechnological Applications

B. popilliae and *B. lentimorbus* have been used effectively for the biological control of the Japanese beetle. The Japanese beetle was so named because it was first known to exist only in Japan. In 1916, Dickerson and Weiss of the New Jersey Department of Agriculture discovered several grubs (larvae) of the Japanese beetle in a nursery in the state of New Jersey, USA. It was speculated (Fleming, 1968) that they were imported in soil surrounding the rhizomes of Japanese iris. Since that time they have spread throughout almost all of the USA east of the Mississippi River. They have also "hitch-hiked" to the northwestern USA, Europe, and Australia.

Japanese beetle larvae eat the roots of grass (yards and pastures), ornamental plants, and garden or truck crops. Adult Japanese beetles eat leaves, flowers, and fruit of trees, ornamental and floral plants, citrus fruits, berry crops, garden vegetables, and farm crops (including corn, soybeans, clover, and alfalfa) (Klein, 1981). In 1982 the total cost for prevention, treatment, and replacement related to turfgrass damage alone in the USA was estimated to be $234 million (Ahmad et al., 1983). This cost does not include the extensive damage caused by the adult beetle, and omits some damage costs caused by the larval form of the beetle.

From 1939 to 1953 a massive program, involving USA federal and state governments, was carried on to accelerate the spread of *B. popilliae* throughout the area infested by the beetle (Fleming, 1968). Adequate biological control of the beetle was attained in much of the area at the time when the program ceased. Serious problems still occur today, however, and the spread of the Japanese beetle to new areas continues.

Spores for the government-subsidized program were produced in vivo; i.e., by the artificial infection of Japanese beetle larvae. Until recently this was also true for commercially available products. Fairfax Biological Laboratory, Inc. (Clinton Corners, NY) continues to obtain their spore preparations from spores generated in vivo. Ringer Corp. (Minneapolis, MN) is producing spores in vitro by use of a patented procedure (Ellis et al., 1989) purchased from Reuter Laboratories, Inc. (formerly of Haymarket, VA). However, their product, Grub Attack®, contains *Bacillus polymyxa* rather than *B. popilliae*. Also, all four strains deposited with the American Type Culture Collection (Rockville, MD) in connection with the patent are not *B. popilliae*. The preferred production strain, ATCC 53256, is *B. polymyxa* and the other strains (ATCC 53257, 53258, and 53259) are *Bacillus amylolyticus* (D. Stahly, unpublished observations).

Other areas of interest relate to the mechanism of pathogenicity and factors regulating host specificity of different strains (Klein, 1981). Research in these areas will undoubtedly aid and strengthen the biotechnological application of these bacteria.

Bacillus larvae

Bacillus larvae was named and described in 1906 by G. F. White, who provided evidence by experimental inoculation that this bacterium caused a fatal disease of honey bee larvae called American foulbrood (White, 1907). Maassen (1906) in Germany and Burri (1904) in Switzerland isolated cultures from larval remains that were named *B. brandenburgiensis* and *B. burri* (Cowan, 1911), respectively. These cultures later proved to be identical to White's isolate. Nomenclatural priority has been given to White. The only known host for *B. larvae* is the honey bee, *Apis mellifera* Linnaeus.

The use of the term "American" in "American foulbrood" is somewhat misleading because the disease occurs throughout the world. The term "foulbrood" is derived from the odor of decaying larvae (brood), described as resembling that of burned glue.

American foulbrood is a very serious disease in part because the bacteria sporulate in the larval remains so that the spores [more than 10^9

spores per larva (Holst, 1946; Shimanuki, 1980)], which are stable for at least 35 years (Haseman, 1961), are left to infect other larvae. When a colony is determined to be infected, government regulations require that the colony be destroyed (most commonly accomplished by burning). Shimanuki (1980) estimated that about 3% of all colonies inspected in the USA are infected with American foulbrood. The direct loss to U.S. apiaries from lost honey and beeswax (based on a total annual value of these products of $132 million [reference is not an exact match Levin, 1984]) is about $4 million. This is a minor part of the total loss, however. It was estimated that in 1985 the value of the increased yield and quality of U.S. crops due to honey bee pollination was $9.3 billion (Robinson et al., 1989a, 1989b). Accordingly, the value of the lost pollination (3%) would be $279 million.

Habitats

The major source of *B. larvae* is the larval remains of honey bees in honeycombs. In a hive infected with American foulbrood, spores of *B. larvae* can also be isolated from the honey, wax, pollen, and hive walls (Gochnauer, 1981; Shimanuki and Knox, 1988).

Isolation, Growth, Maintenance, and Preservation of Cultures

Usually, *B. larvae* can be easily isolated from larval cadavers, since they commonly are present as almost pure cultures (Holst, 1945; Sturtevant, 1924). The relative lack of other organisms suggested to Holst (1945) that *B. larvae* might produce an antibiotic. Indeed, he found that one or more antibiotics were produced during sporulation that were effective against a variety of Gram-positive and Gram-negative species.

Sturtevant (1924) isolated pure cultures from diseased larval cadavers simply by suspending a small amount of the dried remains in water and then streaking a loop of the suspension onto a solid medium.

Rose (1969) developed a method for isolating *B. larvae* from a mixture of other bacteria, including spore-formers. His method is based on the fact that *B. larvae* spores, unlike those of most other sporeformers, do not readily germinate in nutrient broth (Rose, 1969; White, 1906). A few drops of an aqueous suspension of dried larval cadavers were added to a tube of nutrient broth and the tube was heated at 70°C for 15 min. Then it was incubated at 37°C for 12 h. Two additional heat treatments with an intermediate incubation were used before plating on a medium permitting germination of *B. larvae* spores and growth.

Shimanuki and Knox (1988) developed a method for determining the number of *B. larvae* spores in honey. Problems with honey are its high viscosity and the presence of antibacterial substances. Their procedure, in brief, involved heating the honey at 45°C to decrease its viscosity, diluting with water, dialysis, centrifugation, resuspension of the pellet in water, heating at 80°C for 10 min to kill vegetative cells, and plating on a solid medium. By use of this method they showed that *B. larvae* spores were present in 30% of beekeeper-packed honey and 100% of commercial-packed honey (blended from many sources).

Spores or vegetative cells of *B. larvae* can be grown on a variety of solid or liquid media. The medium used by Gordon et al. (1973) was the J-medium (St. Julian et al., 1963), described above for growth of *B. popilliae* and *B. lentimorbus*. Another medium that is satisfactory for the routine growth of *B. larvae* is "modified MYPGP medium", similar to the MYPGP medium described above.

Modified MYPGP Medium for Growth of *Bacillus larvae* (Dingman and Stahly, 1983)

Mueller-Hinton broth	10 g
Yeast extract	15 g
K_2HPO_4	3 g
Glucose (autoclaved separately)	2 g
Sodium pyruvate	1 g
Distilled water	to 1,000 ml

This medium may be solidified by inclusion of 20 g of agar.

When these or other solid media are used for determination of spore count, cultures are first heated at 65°C for 15 min to kill vegetative cells (Dingman and Stahly, 1983; Rose, 1969). A problem with spore counts is the low plating efficiency observed. Dingman and Stahly (1983) reported that heat-resistant counts were usually about 6% of the direct microscopic spore counts. The explanation for this discrepancy is unknown. Heat shocking of spores of *B. larvae* NRRL B-3650 at 60–80°C before plating promoted little to no increase in plating efficiency (Dingman, 1983).

B. larvae cultures can be maintained by growth on either of the above agar solidified media at 30–37°C for 2–3 days. Plates or slants are stored at 4°C and transferred every 2 weeks.

Frozen spore stocks are the most convenient for long-term culture preservation. Most *B. larvae* cultures exhibit low percentage sporulation when grown on J-agar or modified MYPGP agar at 37°C. Vegetative cells can be preserved by conventional lyophilization or by suspension in a freezing medium (e.g., 70-ml Hanks' 1 × medium, 10-ml rabbit serum, 20 ml glycerol) and storage at −70°C.

Identification

No phylogenetic or molecular genetic studies have been performed on *B. larvae* other than an analysis of the nucleotide composition of its DNA; the type strain DNA has a GC content of 50 mol%, based on buoyant density (Nakamura, 1984).

Phenotypic observations indicate that *B. larvae* belongs in the genus *Bacillus* (Claus and Berkeley, 1986). The cells are rod shaped, and endospores are formed within swollen sporangia. No parasporal inclusions have been observed. The vegetative cells have a typical Gram-positive cell wall structure (Bakhiet and Stahly, 1985b). The endospores have a smooth surface, and the inner spore coat is somewhat unusual in that it consists of seven distinct lamellae (Bakhiet and Stahly, 1985b; Bulla et al., 1969).

Some strains are motile by peritrichous flagella. "Giant whips" are often microscopically observed in these strains (Gochnauer and L'Arrivee, 1969a; White, 1907). These were thought to be due to flagella actively discharged from the cells and in an aggregated state (Frank and Hoffman, 1968; Gochnauer and L'Arrivee, 1969a). Ludvik et al. (1983) observed by electron microscopy that in old sporulating cultures cell wall material separates from the cell surface in the form of thick filaments. They observed that the "giant whips" formed when free flagella aggregated with this material.

Other characteristics that are often of value in speciation are type of cell wall peptidoglycan (Schleifer and Kandler, 1972), cellular lipid composition (Kaneda, 1977), and major type of quinone present (Collins and Jones, 1981). Nothing is known about the type of cell wall peptidoglycan possessed by *B. larvae*. Based on lipid composition, Kaneda (1977) placed *B. larvae* in group B (out of six groups in the genus *Bacillus*) together with *B. popilliae*, *B. lentimorbus*, and *B. polymyxa*. The major quinone possessed by *B. larvae* is a menaquinone with seven isoprene units (MK-7). A minor amount of MK-3 is also present (Hess et al., 1979).

Bacillus larvae is similar to *B. popilliae* and *B. lentimorbus* in that it is fastidious. It does not survive serial transfer in nutrient broth. It is a facultative bacterium but it grows much better aerobically (Dingman and Stahly, 1984; Lochhead, 1928). Similar to *B. popilliae* and *B. lentimorbus*, *B. larvae* lacks catalase activity, as measured by observation of oxygen bubbles when a small drop of 10–30% H_2O_2 is applied to the colonies (Haynes, 1972). The catalase test is probably the most helpful diagnostic tool to use after initial isolation of the microbe, in addition to colony appearance.

The many other phenotypic characteristics of *B. larvae* that differentiate it from other *Bacillus* species are reviewed by Claus and Berkeley (1986) and will not be discussed here except as they relate to possible varietal diversity within the species. Jelinski (1985) conducted a study of biochemical reactions of 110 strains of *B. larvae* from five sources. The strains were uniformly positive or negative for most tests. They were variable for nitrate reduction (58% of strains positive), acid production from mannitol (17% positive), and acid production from salicin (11% positive). If denitrification is actually occurring in the positive strains, then the difference between the positive and negative cultures might be sufficient to at least consider varietal differentiation. However, it has not been established that denitrification occurs in the positive strains. No published studies have indicated whether better growth occurs anaerobically in the presence or absence of nitrate. No studies have indicated whether nitrous oxide or nitrogen, products that normally result from denitrification, are produced by the positive strains. The other variable traits may or may not be significant from the perspective of a genetic relationship. Obviously, GC content and nucleic acid hybridization data are needed.

Immunological studies have been performed by several investigators. Antisera against *B. popilliae* and *B. lentimorbus* showed some cross-reaction in agglutination assays with *B. larvae* (Hrubant and Rhodes, 1968). Giauffret et al. (1970) demonstrated some cross-reaction in precipitation and immunofluorescence between *B. larvae* and *B. alvei*. Cross-reaction between *B. larvae* and *B. alvei* was not observed in immunodiffusion and immunofluorescence assays conducted by Peng and Peng (1979). Otte (1973) reported that antiserum against *B. larvae* produced little to no cross-reaction with 36 strains of other bacteria by immunofluorescence. Preliminary results of Giauffret et al. (1970) indicated that there are some important serological differences between strains of *B. larvae*, but more detailed investigations are needed to determine if varietal differentiation is justified.

Bacteriophage typing has been employed for varietal differentiation of *B. thuringiensis* and *B. sphaericus* strains. Several temperate phages of *B. larvae* have been isolated by different investigators (see "Genetics," this chapter). Some strains of *B. larvae* are sensitive, and some are immune (or resistant). Some of the strains are immune simply because they possess the same or related phages in the prophage state (Bakhiet and Stahly, 1988; Dingman et al., 1984; D. P. Stahly, unpublished observations). It is not clear, however, whether some of the cases of resistance are an indication of more basic cellular differ-

ences between *B. larvae* strains. More research is required before it can be determined whether phage typing will be of value for varietal differentiation.

Ecophysiology

NUTRITION. As stated previously, *Bacillus larvae* is fastidious, failing to grow on nutrient agar. This characteristic led early investigators to supplement their usual media with many different natural substances: e.g., macerated, healthy bee larvae (White, 1906); calf or pig brain extract (Maassen, 1908); egg yolk (White, 1919); egg yolk and yeast (Sturtevant, 1924); yeast extract and carrot extract (Lochhead, 1933); and minced chicken embryo (Tarr, 1937a). In 1942 Lochhead found that thiamine replaced all of these special requirements. His basic medium consisted of glucose, peptone, thiamine, K_2HPO_4, and various salts. Foster et al. (1950) found that poor growth occurred in the complex medium of Lochhead (1942) unless it was pretreated with charcoal, supplemented with starch, or a combination of the two. Their hypothesis is that anti-growth substances were removed or sequestered from the medium. Katznelson and Lochhead (1948) attempted to develop a synthetic medium for *B. larvae*. In addition to thiamine, a purine, either xanthine or guanine, was required. However, when a basal salts-glucose medium was supplemented with 18 amino acids, thiamine, and a purine, growth sufficient for serial transfer only occurred when the medium was made semisolid with agar.

B. larvae is capable of catabolizing the carbohydrates glucose, fructose, galactose, trehalose, glycerol, mannose, and ribose. Use of mannitol, xylose, sucrose, and salicin is strain variable (Azuma and Kitaoka, 1965; de Barjac, 1981; Jelinski, 1985; Lochhead, 1928).

B. larvae is facultative but grows much better in the presence of oxygen (Bailey, 1968). Bailey and Lee (1962) inoculated tubes containing a semisolid medium with spores of *B. larvae* and, after incubation, observed growth in a band (sometimes two bands) 5–10 mm below the surface. The authors concluded that the spores germinated and grew best under microaerophilic conditions. Dingman (1983) observed similar banded growth after inoculating semisolid medium with vegetative cells. The evidence of Bailey and Lee (1962) may not relate to the oxygen requirement for germination, as claimed, since it is possible that germination occurs at positions in the tubes other than where growth occurs.

GROWTH AND SPORULATION IN VIVO. Larvae of honey bees become infected by ingestion of spores of *B. larvae*. Spores are the only infective form (Tarr, 1937b), probably because vegetative cells are rapidly killed by royal jelly, the food material in which the larvae are immersed (Holst, 1946). When vegetative cells of *B. larvae* were suspended in royal jelly (10^7 cells/ml), < 10 viable cells/ml remained after 30 s at room temperature (N. Bakhiet and D. P. Stahly, unpublished observations). The bactericidal effect, due primarily to 10-hydroxy δ^2-decenoic acid (Blum et al., 1959), is apparently diminished or absent in the midgut, where the spores germinate and grow.

The larvae are very susceptible to infection only up to an age of about 1.5 days. After 1.5–2 days of age they become totally resistant (Bamrick, 1964; Bamrick and Rothenbuhler, 1961; Hoage and Rothenbuhler, 1966). Davidson (1973) studied the histopathology of American foulbrood and in the process discovered a possible mechanism for the age-related development of resistance. She determined that vegetative cells in the midgut lumen invaded the peritrophic membrane by a phagocytosis-like process, traversed the epithelium, and entered the hemocoel, where they grew to high populations and caused a generalized septicemia. The first barrier, the peritrophic membrane, although present in larvae as young as 8 h, increases tremendously in thickness and "density of composition" with age (Davidson, 1970, 1973). Davidson suggested that this structural change may contribute to the age-related resistance. Nothing is known about the mechanism of attachment of *B. larvae* cells to the peritrophic membrane that must precede phagocytosis. Perhaps the glucose, mannose, and galactose-containing capsular polysaccharide (Li et al., 1985) is involved.

Death usually occurs at an age of 8 to 11 days; 2 days before or after the transition to the pupal stage. As stated above, death is preceded by growth in the hemolymph to high populations and a general invasion of most body tissues. Davidson (1973) reported heavy infection of the fat body and rupture of hemocytic membranes. The dead larvae rapidly decompose as *B. larvae* exhibits massive sporulation.

The question of whether a toxin or an enzyme of *B. larvae* is involved in pathogenicity is an open one. Aqueous extracts of cadavers of larvae killed by American foulbrood are toxic by feeding to honey bee larvae (Patel and Gochnauer, 1959). Such larval remains have a high level of protease activity (Holst and Sturtevant, 1940; Patel and Gochnauer, 1959), suggesting that protease activity and toxicity are related. Heating of the extract for 1 min at 100°C inactivated both protease activity and toxicity (Patel and Gochnauer, 1959). Holst and Sturtevant (1940) demonstrated protease activity only in sporulat-

ing cultures of *B. larvae*. Furthermore, certain asporogenic mutants did not produce protease. These facts led Bamrick (1964) to question involvement of proteases in the disease process; he suggested that their involvement was probably in postmortem decomposition, a process that occurred simultaneously with the appearance of spores. Gochnauer (1969) and Patel and Gochhauer (1972) separated multiple (two to three) proteases from cells at different stages of development. One enzyme, stated to be associated with "vegetative cells," was obtained from cells in cultures that were 3–10 days old; probably stationary-phase cells. Protease excretion by other *Bacillus* species occurs early in the sporulation process, at a time coincident with the beginning of the stationary phase (reviewed by Freese and Heinze, 1983). Since proteases are normally excreted early in sporulation, it is possible that proteases might be involved in late pathological changes in larvae, even before refractile spores are visible. The availability of a protease-minus, spore-plus mutant would aid in analyzing this question. Proteases are not involved in the initial, early penetration of *B. larvae* of the peritrophic membrane and epithelial cells. This conclusion is supported by the observation of Davidson (1973) that penetration of the peritrophic membrane is not accompanied by physical evidence of enzymatic digestion of the membrane.

Sporulation In Vitro. Whereas *Bacillus larvae* sporulates efficiently in the hemolymph and other tissues of bee larvae, most strains sporulate very poorly in or on artificial media. The study of American foulbrood has been slowed somewhat by the difficulty of obtaining large numbers of spores in vitro. Interest about in vitro sporulation is heightened by the possibility that what is learned about sporulation in *B. larvae* may, in part, be applicable to the other catalase-minus insect pathogens, *B. popilliae* and *B. lentimorbus*.

Sporulation has been reported to occur at a higher frequency in cells grown on solid media rather than in broth media. A variety of solid media has been used which promotes sporulation of *B. larvae* (Azuma and Kitaoka, 1965; Bailey and Lee, 1962; Dingman and Stahly, 1983; Foster et al., 1950; Gordon et al., 1973; Lochhead, 1928; Smith et al., 1949). Azuma and Kitaoka (1965) indicated that they obtained sporulation by growth on slants in tubes "closed tightly with rubber stoppers"; an indication that the sporulation process may be somewhat oxygen-sensitive.

Gochnauer (1969) and Gochnauer and L'Arrivee (1969a) used a biphasic system to obtain sporulation. The bottom phase consisted of Difco brain-liver-heart agar. The top phase was a shallow layer of broth medium of the same components as the bottom phase (except agar) or a yeast extract, starch, and potassium phosphate medium (Bailey and Lee, 1962). Cultures were incubated at 35°C for 5–10 days without shaking.

There are only three reports of fair-to-good sporulation in single-phase, liquid media. St. Julian and Bulla (1971) obtained 2×10^7 spores/ml of the type strain, *B. larvae* NRRL B-2605, in liquid MD medium, which contains yeast extract, glucose, and K_2HPO_4. Gochnauer (1973) obtained 10^8 spores/ml after incubation with gentle shaking of *B. larvae* 34A in brain heart infusion broth plus thiamine hydrochloride. Dingman and Stahly (1983) obtained 5×10^8 spores/ml after incubation with gentle shaking of *B. larvae* NRRL B-3650 in TMYGP broth, which contains tris-maleate buffer, yeast extract, glucose, and sodium pyruvate. The level of aeration was critical to achieve sporulation of strain B-3650; aeration was required, but at a level much reduced from that used to obtain sporulation of more typical *Bacillus* species. This finding is consistent with the observation of Fitz-James and Young (1969) that sporulation of *B. larvae* was inhibited by excess shaking.

Lodesani et al. (1985) used a rather nontraditional method to obtain sporulation of strains that sporulate poorly if at all on solid media. A culture was grown in a broth medium, cells were concentrated by centrifugation and suspension in a small volume of phosphate buffer, and the suspension was placed in a sterile dialysis bag suspended in a broth medium containing yeast extract, potassium phosphate, and starch. The culture was incubated without shaking at 34°C for 30 days. The authors stated that, depending on the strain, 10–80% of the cells introduced into the dialysis bags sporulated.

Metabolism. Aspects of metabolism discussed below deal with oxygen metabolism, protection against toxic forms of oxygen, pathways of glucose catabolism, and nutrient requirements for sporulation.

Bacillus larvae, when grown in the presence of air, consumes oxygen as would be expected (Dingman and Stahly, 1984). Most oxygen-utilizing bacteria possess superoxide dismutase to protect against superoxide (O_2^-) and catalase and/or peroxidase to protect against H_2O_2. *B. larvae* NRRL B-3650 resembles *B. popilliae* and *B. lentimorbus* in that it has superoxide dismutase levels approximately equal to those of *B. subtilis* 168, a "typical" *Bacillus* species, but lacks NADH peroxidase and has very low catalase activity (Dingman and Stahly, 1984). Catalase activity is nondetectable during growth. It appears during the early stationary phase and

increases during the time of appearance of refractile spores. The activities at these two stages are about 2,700 and 5,400 times less than those exhibited by *B. subtilis*. The relative absence of catalase (and NADH peroxidase) may be the cause of the apparent oxygen-sensitivity of sporulation (Dingman and Stahly, 1983). *B. larvae* does not accumulate H_2O_2 extracellularly, but intracellular accumulation remains a possibility (Dingman and Stahly, 1984).

Mylroie and Katznelson (1957) reported that *B. larvae* metabolizes glucose primarily to acetate during the exponential growth phase. They demonstrated the presence of enzymes of both the Embden-Meyerhoff-Parnas pathway and the pentose phosphate pathway. In an extension of this work, St. Julian and Bulla (1971) showed that key enzymes of the Entner-Doudoroff pathway, as well as those of the other two pathways, are present in *B. larvae* NRRL B-2605. This is rather unusual since the Entner-Doudoroff pathway has not been demonstrated in other *Bacillus* species, and the pathway rarely occurs in Gram-positive bacteria. Radiorespirometric analysis, however, indicates that the Entner-Doudoroff pathway is not being used to a significant extent; the pentose phosphate pathway is the primary pathway used.

No information exists on how glucose is transported into cells; no phosphoenolpyruvate: glucose phosphotranferase activity has been detected (St. Julian and Bulla, 1971). Also, no information exists on the mechanism of uptake and catabolism of trehalose, the main carbohydrate in honey bee larval hemolymph.

St. Julian and Bulla (1971) showed that glucose degradation by *B. larvae* NRRL B-2605 occurs during growth, resulting in a decline in pH due to acid (probably acetic acid) accumulation. When growth ends coincident with depletion of glucose from the medium, the pH increases. This pH increase is due to complete oxidation of acetate through the citric acid and glyoxylic acid cycles.

The pattern of glucose utilization by *B. larvae* NRRL B-3650 is surprisingly different from that described above for the type strain (NRRL B-2605) (Dingman and Stahly, 1983). Strain B-3650 does not degrade glucose during growth; glucose oxidation begins at the end of exponential growth. The only component of TMYGP broth required for growth of strain B-3650 is yeast extract. Acids were produced during growth, presumably from yeast extract components of the TMYGP broth (see "Ecophysiology; Sporulation In Vitro," this chapter). Thus, glucose usage in this strain may be regulated by a type of catabolite repression. Another unusual property of strain B-3650 is that both glucose and pyruvate are required for sporulation. In most

Bacillus species glucose represses sporulation. Glucose is needed by strain B-3650 for a late event(s) in sporulation; it could be added as late as 35 h (25 h after the end of exponential growth) without any decline in the number of spores formed. (Spores are first apparent at 45 h.) This requirement can be partially satisified by substitution of glucosamine or potassium gluconate, but not by galactose, fructose, mannitol, or glycerol. Why pyruvate is required for sporulation is not readily apparent. Pyruvate is depleted from the medium before sporulation begins. Substitution of acetate, lactate, citrate, glutamate, or succinate does not satisfy this requirement (Dingman, 1983; Dingman and Stahly, 1983).

Genetics

Bacillus larvae exhibits instability with regard to sporulation ability and virulence. Tarr (1937b) reported that serial transfer on an artificial medium resulted in loss of ability to sporulate. Holst and Sturtevant (1940) observed frequent asporogenic sectors in colonies. Stahly and Livasy (unpublished observation) have made similar observations with *B. larvae* NRRL B-3650. Asporogenic colonies remained asporogenic; i.e., the change was not reversible. Shimanuki et al. (1965) indicated that virulence of *B. larvae* was increased by passage three times through honey bee larvae. To understand these and other phenomena, an understanding of the genetics of *B. larvae* is highly desirable.

Conditions have been developed for polyethylene glycol-mediated plasmid transformation and phage DNA transfection of protoplasts (Bakhiet and Stahly, 1985a; Chen and Yin, 1986). Although several phages have been isolated from *B. larvae* (see below), no reports of transduction exist. Also, there have been no reports of conjugation.

Three morphologically distinct temperate bacteriophages have been isolated from *B. larvae*. PBL1, originally isolated from *B. larvae* NRRL B-3553 by Gochnauer (1955, 1970), has been purified and characterized (Dingman et al., 1984; Gochnauer and L'Arrivee, 1969b). Based on comparative electron microscopic data, phage BLA, isolated by Drobnikova and Ludvik (1982), is a PBL1-like phage. PBL1-like phages are very common in *B. larvae* strains isolated from different geographical locations within the USA (D. P. Stahly, unpublished observations). Another temperate phage, PBL0.5, was isolated from strain NRRL B-3558 (Dingman et al., 1984) and characterized (Bakhiet and Stahly, 1988). An apparently identical phage, PBL2, was isolated from strain NRRL B-3553, where it was found to coexist in the prophage state with PBL1 (Bakhiet and Stahly, 1988). It is probable that

phage BL2, isolated by Benada et al. (1984b), is a PBLO.5-like phage; a conclusion based on comparative electron microscopic data. A morphologically distinct temperate phage, PBL3, was isolated from *B. larvae* GA and was partially characterized (Campana, N. Bakhiet, and D. P. Stahly, unpublished observations). It is hoped that these phages will be of value for phage typing and, perhaps, for development of a transduction system(s). None of the phages PBL1, PBL0.5, and PBL3 produced plaques on a variety of other *Bacillus* species, including *B. popilliae* (H.-W. Ackermann, personal communication; D. Takefman and D. P. Stahly, unpublished observations).

The only published information on plasmids in *B. larvae* is that of Benada et al. (1984a). They found cryptic plasmids in three of 12 strains tested.

Preliminary evidence suggests that a restriction-modification system exists in strain NRRL B-3555 (Bakhiet and Stahly, 1985).

Bacillus thuringiensis

Bacillus thuringiensis is a Gram-positive, facultative, sporeforming, rod-shaped bacterium that has been the subject of intense investigation in laboratories worldwide. As a member of the genus *Bacillus*, the organism shares with the other members of the taxon the ability to form endospores that are resistant to inactivation by heat, desiccation, and organic solvents. Biochemically, sporulation in *B. thuringiensis* is very similar to that in other sporeforming bacteria and the spores resemble those of other bacilli in morphology and composition. However, the formation of one or more parasporal crystalline bodies adjacent to the spore during stages III to V of sporulation distinguishes *B. thuringiensis* from most other *Bacillus* species (Andrews et al., 1982, 1987; Bulla et al., 1985).

B. thuringiensis is probably best known as an insect pathogen. Older cultures, which contain spores and parasporal crystals, are highly toxic to larvae of members of certain insect orders. This property has caused extensive interest in this organism because of the potential to use it to formulate more effective, specific, and environmentally safe insecticides (Bulla et al., 1985).

Although *B. thuringiensis* is capable of saprophytic existence in soil, it is also a frank insect pathogen. Not surprisingly, the initial discovery of *B. thuringiensis* was as an insect pathogen, since its pathogenicity made its existence in the environment more obvious. Probably the first description of *B. thuringiensis* was by Ishiwata, who, in 1901, described the etiological agent of "sotto disease" or "flacherie." Signifi-

cantly, Ishiwata was also the first to describe the relationship between the culture's age and pathogenicity. Young cultures of the "sotto disease bacillus," as he termed his isolate, were not as pathogenic to larvae of the Japanese silk moth, *Bombyx mori,* as were older cultures (Bulla et al., 1980; Ishiwata, 1901).

Probably the first to suggest the use of *B. thuringiensis* for controlling insect pests was Berliner (1911, 1915), who, apparently independently of Ishiwata, described the causative agent of a disease of the Mediterranean meal moth, *Anagasta kuhniella.* Berliner, and later Mattes, described the crystalline inclusion body, or "Restokorper," within sporulated cells, and it was Berliner who first used the species name *thuringiensis,* deriving the name from the German province of Thuringia (Bulla et al., 1980).

As reviewed by Andrews et al. (1987), from the initial discovery of the microbe until the early 1950s, numerous studies appeared in the literature aimed at using *B. thuringiensis* in insect control without sufficient basic information to accomplish these goals. In the early 1950s, investigators began studying the basic biology of *B. thuringiensis* and considering some fundamental properties of its insect pathology. Steinhaus (1951), for example, published an electron micrograph of the crystal toxin, and in 1953 Hannay described the development of the crystal toxin in relation to the sporulation cycle. Hannay (1953) and Hannay and Fitz-James (1955) observed that the diamond-shaped crystals or parasporal bodies are composed primarily of protein and suggested that the crystals might be involved in the establishment of septicemia in insect larvae. Angus (1956a, 1956b) prepared toxic filtrates that included crystals alone and crystals plus spores and showed that the crystals were responsible for midgut paralysis and cessation of feeding, whereas the spores were required for septicemia.

Habitats

Bacillus thuringiensis is a member of a limited group of bacteria that occurs both naturally and can be added to an ecosystem to achieve insect control. A somewhat arbitrary distinction is made between "natural" and "artificial" habitats of *B. thuringiensis*. A habitat is defined as natural when *B. thuringiensis* can be isolated when there is no previous record of application of the organism to that ecosystem. A habitat is considered artificial, on the other hand, when there has been previous application of the organism for insect control. It is important to note that, in the latter case, artificial and natural may not be mutually exclusive; *B. thuringiensis* may occur naturally in

environments where additional organisms have been applied.

Most *B. thuringiensis* cultures that have been studied were either isolated directly from infected insects or from soil associated with them. The spores of *B. thuringiensis* readily persist in soil and vegetative growth occurs when nutrients are available. All of the early isolates were pathogenic for insects and this pathogenicity became recognized as a differentiating characteristic of the species, in addition to possession of parasporal bodies. However, it is now apparent that *B. thuringiensis* isolates can be obtained from soil that has not been exposed to *B. thuringiensis*-derived insecticides and in which susceptible insects are not abundant. Moreover, many of these isolates are nontoxic for all insects tested. For example, Ohba and Aizawa (1986) isolated 189 cultures of *B. thuringiensis* from 136 soil samples collected from nonsericultural areas of Japan. The classification was based in part on possession of parasporal bodies (and not insect pathogenicity). According to Ohba et al. (1988), the majority of such *B. thuringiensis* isolates are nontoxic for a variety of insects.

De Lucca et al. (1981) conducted a similar survey of the presence of *B. thuringiensis* in soil in the USA. They found that *B. thuringiensis* represented between 0.5 and 0.005% of all *Bacillus* species isolated from the soil samples. Travers et al. (1987) screened soil samples collected from random locations in Montgomery County, MD, USA, for the presence of *B. thuringiensis*. It was found in almost all of the soil samples examined. Thus, it is obvious that *B. thuringiensis* is widespread in nature.

One might expect that epizootics caused by *B. thuringiensis* would be frequent, considering the widespread occurrence of the species in soil. However, epizootics are somewhat rare. Most epizootics have been limited to situations wherein the insect density is relatively high, providing better opportunity for establishing the disease within the population. In high density infestations of Douglas-fir tussock moth populations, for example, *B. thuringiensis* epizootics become a factor in reducing defoliation and eventual collapse of the population (Tunnock et al., 1974). Similarly, outbreaks of disease in insect populations have been reported in stored grain pests (Burges and Hurst, 1977; Vankova and Purrini, 1979) and in the European corn borer (Lynch et al., 1976). *B. thuringiensis* subsp. *israelensis,* which is a strain highly toxic to larvae of mosquitoes and some other hematophagous-insects (Klowden and Bulla, 1984; Tyrell et al., 1979), was first isolated from pond water in Israel wherein many diseased larvae were observed (Goldberg and Margalit, 1977; Margalit and Dean, 1985).

The artificial habitats are the locations where *B. thuringiensis* insecticides (usually a mixture of spores and crystals) are applied. Insecticides formulated with *B. thuringiensis* are currently being manufactured and used worldwide. Major producers in the USA, Europe, and the USSR make insecticides formulated with *B. thuringiensis*. Bulla et al. (1985) defined two groups of insecticides formulated with *B. thuringiensis*. Group I consists of products formulated with strains having toxicity against lepidopteran insects, whereas group II products are those with toxicities similar to *B. thuringiensis* subsp. *israelensis,* which are highly toxic to mosquitoes and certain other hematophagous dipteran larvae. With the discovery of strains such as subsp. *tenebrionis,* which are toxic to coleopteran larvae, a group III needs to be defined. Accordingly, *B. thuringiensis* may be applied as an insecticide on foliage, in soil, in water environments, and in food storage facilities (e.g., grain bins).

Isolation, Preservation, and Growth of Cultures

Isolation of *B. thuringiensis* from soil or other natural environments is greatly facilitated by use of selective techniques. Travers et al. (1987) took advantage of the observation that germination of spores in crystal-forming bacilli, including both *B. thuringiensis* and *B. sphaericus*, is inhibited by sodium acetate concentrations of approximately 0.25 M. Soil, which contained up to 10^9 bacteria/g, was inoculated into a nutrient medium that contained the sodium acetate. After a period of growth, the vegetative cells were eliminated by heat treatment and the remaining spores were isolated on a nutrient medium without acetate. The survivors from this treatment ranged from 20–96% *B. thuringiensis* and/or *B. sphaericus*. These two species are easily differentiated by observation of colonial and cellular morphology. Similarly, Saleh et al. (1969) exploited the observation that *B. thuringiensis* is relatively resistant to polymyxin B and penicillin G to isolate *B. thuringiensis* from soil.

Preservation of *B. thuringiensis* cultures is of special importance because of the common presence of multiple plasmids, some of which carry vital information; e.g., the crystal toxin gene (Faust et al., 1979; reference is not an exact match Gonzales et al., 1982). These plasmids may be lost during routine subculturing, and, therefore, crystal toxin production and, presumably, other plasmid-borne phenotypes may be simultaneously lost (Stahly et al., 1978). Investigators in R.E. Andrews' laboratory use three methods for culture preservation.

For long-term culture storage, cultures are grown until sporulation occurs (about 24 h) in

liquid GYS medium (Nickerson and Bulla, 1974). After >90% of the cells in the culture contain mature, phase-bright spores, the cells are harvested by centrifugation and resuspended in 20% skim milk. This material is then lyophilized by standard techniques (Gherna, 1981). For routine laboratory operations, several agar slant cultures are prepared at the same time by inoculation with the *B. thuringiensis* culture to be preserved. These are incubated at 30°C for 4 days to allow extensive sporulation and then stored at 4°C for up to 6 months. When a culture is required for an experiment it can be started from one of these stored slants, which are used only once after they are removed from cold storage. The slant methodology has certain disadvantages. The inoculum contains frequently variable amounts of spores and the culture outgrowth can be somewhat erratic. For synchronized sporulation of *B. thuringiensis*, another method is used. The culture is grown to midlogarithmic phase in liquid modified GYS medium ($A_{600} = 0.5$) and then diluted 1:50 in modified GYS medium containing 50% (vol/vol) glycerol. This material is divided into 100-μl portions and stored in sterile Eppendorf test tubes at −70°C. Viability remains high for up to 1 year.

B. thuringiensis is not particularly fastidious and can be routinely cultured on many complex media; e.g., Difco nutrient agar, LB agar (reference is not an exact match Maniates et al., 1982), and Difco brain heart infusion agar. Although *B. thuringiensis* is facultative, it grows best aerobically. For growth in liquid media, cultures are usually aerated by vigorous shaking of cultures in Ehrlenmeyer or Fernbach flasks. Those in the R.E. Andrews' laboratory use 100 ml of medium in 300 ml Ehrlenmeyer flasks and 1 l of medium in 2.8-l Fernbach flasks. The use of flasks with baffled bottoms is not necessary, but may increase growth rates by about 10% (R. E. Andrews, unpublished observation).

Much of the understanding of sporulation and crystal toxin formation is derived from the observation that *B. thuringiensis* can be grown and induced to sporulate in near synchrony. That is, as the cells begin to sporulate, >99% of the cells will be in the same morphological stage. Bechtel and Bulla (1976), for example, used a medium that contained glucose, yeast extract, citric acid, and salts (modified GYS) to study sporulation and crystal toxin with the electron microscope. In these studies, the parasporal crystals formed in synchrony and allowed a detailed understanding of the morphological events related to sporulation. Later Andrews et al. (1981, 1982, 1985) used these same culture methods to show that crystal antigen first appeared in the cells at a defined time during the sporulation process, and that its synthesis was induced by de novo synthesis of crystal toxin specific mRNA. Moreover, this medium can be used to produce a nearly pure suspension of spores and crystals that can readily be used to purify spores and/or crystals for further use (Tyrell et al., 1981). The methodology works well with all subspecies tested to date (R. E. Andrews, unpublished observations).

Investigators in R. E. Andrews' laboratory currently use the following method to achieve synchronous sporulation without excessively long incubation times. A small volume (usually about 5 μl) of the vegetative cell culture preserved in 50% glycerol at −70° is inoculated into 100 ml of YEG broth (Tyrell et al., 1981). The culture is incubated overnight (about 15 h) at 30°C with vigorous shaking. Some calibration of the starting inoculum is required to insure that the overnight culture does not reach stationary phase. A transfer is made (10% inoculum) to modified GYS medium, and the cells are grown to mid-exponential phase (A_{600}, about 0.5). This culture serves as inoculum for the final experimental culture. Inoculum (10%) is transferred into modified GYS medium. Growth begins immediately with a generation time of 50–60 min, the cells enter stationary phase (the beginning of sporulation) after about 4 h, and sporulation is complete by 12 h. The preliminary transfers result in selection for rapidly growing cells, thus insuring development of physiological and morphological synchrony during sporulation.

Growth requirements for *B. thuringiensis* are relatively simple. Nickerson and Bulla (1974) and Kuznetsov and Khovrychev (1984) described growth and sporulation of several *B. thuringiensis* strains in synthetic media. In addition to basal salts these media, which were similar in composition, contained glucose and glutamate, although some strains required aspartate, citrate, alanine, and/or nicotinic acid. Growth is slower than in complex media, with doubling times often in the range of 3–4 h, but such media should facilitate nutritional studies and selection of auxotrophic mutants.

The conditions used for commercial production of *B. thuringiensis* differ somewhat from those used in the laboratory. Fermentation media usually include unrefined substrates such as corn steep solids, molasses, corn starch, cotton seed flour, fish meal, hydrolyzed casein, groundnut cake, and soybean cake. Although sporulation is probably a prerequisite to crystal toxin, the goal of such fermentations is to optimize the production of crystal toxin rather than to maximize the formation of spores. Typically, fermentation begins with inoculation of a 15-l vessel from a seed culture. This 15-l culture is then used to inoculate larger vessels, and typical final fer-

mentation volumes as large as 30,000 to 100,000 l are common (Andrews et al., 1987).

Identification

MORPHOLOGICAL CHARACTERISTICS. *Bacillus thuringiensis* cells are Gram-positive, spore-forming, and rod-shaped. The cells are straight rods that are typically 1.0–1.2 by 3–5 μm in size when grown in standard liquid media. Terminal to subterminal ellipsoidal spores are formed in sporangia that are not swollen. Probably the most characteristic distinguishing feature of *B. thuringiensis* is the presence of a parasporal crystal that forms adjacent to the spore, outside the exosporium during endospore formation. The presence of the parasporal crystal in cells and the production of insecticidal activity remain the best criteria available for differentiation of *B. thuringiensis* from the closely related species, *B. cereus* (Andrews et al., 1987; Baumann et al., 1984; Claus and Berkeley, 1986).

There is an interesting correlation between the spectrum of toxic action of a given strain and the shape of the crystal it produces. This is discussed in more detail elsewhere in this chapter. For the purpose of this discussion it is important to note, however, that the shape of the crystalline toxin can provide some important clues to the identity of the *B. thuringiensis* isolate in question. Subspecies *kurstaki,* for example, produces a single bipyrimidal crystal, whereas subsp. *israelensis* produces multiple, plemorphic, and globular crystals. If the task at hand is to differentiate between these two subspecies, the observation of crystals by use of a phase contrast microscope will provide meaningful information.

PHYSIOLOGICAL CHARACTERISTICS. Identification of *B. thuringiensis* by physiological characteristics is difficult. Aside from the presence of the crystal toxin, phenotypic characteristics to distinguish between *B. thuringiensis* and *B. cereus* are few. Claus and Berkeley (1986) compared approximately 40 phenotypic characteristics among the bacilli. These two species could not be differentiated by any of these physiological criteria. Baumann et al. (1984) and Lynch and Baumann (1985) examined 137 isolates of *B. thuringiensis* and 35 strains of *B. cereus* for the presence or absence of 99 phenotypic characteristics. Numerical analysis of the data showed that the various subspecies of *B. thuringiensis* (based on flagellar H antigen type) and *B. cereus* did not form two distinct clusters. On the contrary, *B. thuringiensis* and *B. cereus* strains were randomly dispersed throughout the dendrogram. Thus, although identification of an isolate as belonging to the *B. cereus-B. thuringiensis* group can be accomplished by using physiological criteria, other methods are required to distinguish between the two species. Also, a determination of physiological characteristics is of no value in differentiating between the *B. thuringiensis* subspecies.

IMMUNOLOGICAL CHARACTERISTICS. Immunological methods have also been used to identify *B. thuringiensis*. In addition, the basis for the subspecies classification of an isolate is largely based on antigen identification (de Barjac and Bonnefoi, 1962, 1973). Two antigenic targets have been used for immunological identification of *B. thuringiensis*, the flagellar antigens and the crystal toxin protein.

By immunologically analyzing strains for flagellar H antigens, more than 20 subspecies of *B. thuringiensis* have been identified (de Barjac and Bonnefoi, 1962, 1973; Krywienczyk et al., 1978). Although the major criterion for classification of an isolate as *B. thuringiensis* is the presence or absence of the crystal toxin, the use of H antigens has distinct advantages for the identification of subspecies. There is extensive evidence that the crystal toxin gene is frequently located on self transmissible plasmids (Battisti et al., 1985; Gonzales et al., 1982; Green et al., 1989; Klier et al., 1983; Ruhfel et al., 1984; Stahly et al., 1978). Moreover, the crystal toxin gene in many *B. thuringiensis* isolates has been shown to be surrounded by transposon-like DNA sequences (Bourgouin et al., 1988; Lereclus et al., 1984; Lereclus et al., 1986; Mahillon and Lereclus, 1988; Mahillon and Seurinck, 1988; Mahillon et al., 1985, 1987) and, therefore, may be transferable between strains. Thus, because the crystal toxin type produced by a *B. thuringiensis* isolate is probably a relatively inconstant characteristic, use of a more stable antigen as a target for identification is of greater utility. Presumably the flagellar antigen would be of chromosomal origin and would therefore exhibit such stability.

Despite their comparatively unstable nature, crystal toxin antigens have been used for identification of *B. thuringiensis* subspecies. As taxonomic tools, the limitations of these methods have already been described (Krywienczyk, 1977; Krywienczyk et al., 1981). There are, however, some important applications for the use of these antigens; the observation that the crystal toxin presence is intimately correlated with insecticidal activity has been discussed previously. Thus, immunological determination of crystal toxin presence is a useful method for estimating toxic activity of insecticidal preparations. Rocket immune electrophoresis, for example, has been used to determine the toxin content in cultures of *B. thuringiensis* during growth and

sporulation (Andrews et al., 1980, 1981, 1985). This method is rapid and simple, and it reliably estimates the toxic activity of cultures (Andrews et al., 1980). Immunological methods can also be used to quantify crystal toxin in the environment, and they are useful, therefore, for determination of residual insecticidal activity after pesticide application. Enzyme-linked-immunosorbent assay (ELISA), for example, can be used to determine crystal antigen with reasonable sensitivity even under field conditions (Wie et al., 1982, 1984).

In addition to quantification of insecticidal activity, there is another important use for immunological measurement of crystal toxin antigens. Immunological methods provide qualitative information about the crystal toxin. Wie et al. (1982) prepared antibody directed against the crystal toxin of an isolate of subsp. *kurstaki,* and then used heterologous crystal toxin proteins from several other subspecies to inhibit the homologous reaction in ELISAs. Of the five heterologous crystal types examined, four were toxic to lepidopteran larvae, and all four of these were highly inhibitory to the toxin antitoxin homologous reaction. As discussed below, DNA sequence data show that the toxin genes of lepidopteran-toxic strains of *B. thuringiensis* exhibit considerable homology. Tyrell et al. (1981) prepared sera against several crystal toxins from different subspecies and, by using Ouchterlony double diffusion assays, were unable to demonstrate cross-reactivity between the lepidopteran-toxic crystal antisera and the subsp. *israelensis* crystal toxin antigens. Interestingly, although no cross-reactivity was demonstrated with the Ouchterlony assays, Wie et al. (1982) found a low but significant (about 10%) inhibition of the subsp. *kurstaki* homologous reaction when the subsp. *israelensis* crystal protein was used in ELISA studies. The significance of this inhibition did not become clear until DNA sequencing studies revealed that these two toxins shared some similarity (Thorne et al., 1986). Therefore, immunological techniques employing crystal toxin antigens may have an important role in the regulatory process regarding pesticide registration. Under U.S. law, the Office of Pesticide Regulation of the Environmental Protection Agency has a responsibility to ensure that the toxin in a pesticide is the same as that contained therein at the time of the initial registration. Given the genetic exchange capacity of *B. thuringiensis*, these methods allow the registrant to characterize, in a standard manner, the active ingredient of their product, and to ensure that the current production strain expresses the same toxins and toxicity as when it was first registered.

GENETIC CHARACTERISTICS. Characterization of an isolate's DNA content is an important method for identifying *Bacillus thuringiensis*. The DNAs from *B. thuringiensis*, *B. anthracis*, and *B. cereus* are highly homologous, exhibiting >90% homology (Kaneko et al., 1978). Even though these other two species are common in soil, it should still be possible to use DNA probes to identify an isolate as *B. thuringiensis* by using a crystal toxin gene as a probe to detect homologous DNA target sequences in the test strain (Prefontaine et al., 1987). Use of such a DNA probe would obviate the need to detect the gene product, crystal toxin. As previously described, the major criterion for classification of an isolate as *B. thuringiensis* is the presence of crystal toxin. It is possible, however, that a crystal toxin gene may be present but not expressed. A mutation in the toxin gene could prevent expression. Klier and Lecadet (1976) first suggested and then Brown and Whiteley (1988) confirmed that a unique form of RNA polymerase is required for transcription of the crystal toxin gene in *B. thuringiensis*. Subsequently, Adams et al. (1989) have shown that a 20-kDa protein is required for expression of the crystal toxin gene. Mutations that effect expression of genes encoding one of these required components would create a strain that is unable to produce crystal toxin but contains an intact crystal toxin gene. It is unclear at present whether such mutants occur in nature, but their presence would certainly further confuse the taxonomic data.

Plasmid analysis can be used to identify specific isolates within the species *B. thuringiensis*. Most isolates of *B. thuringiensis* contain multiple plasmids ranging from as small as 1–2 kb in size to over 100 kb. Moreover, many isolates have unique plasmid size profiles that can be used in strain determination (Carlton and Gonzalez, 1985; Gonzales et al., 1982; Kronstad et al., 1983; Mahillon et al., 1988; Stahly et al., 1978; Stepanova and Azizbekian, 1987). Because of the potential loss of plasmids during extended culturing (Dean, 1984; Stahly et al., 1978) this method may be of somewhat limited utility as well.

Ecophysiology

Most evidence seems to indicate that the disease symptoms caused by *B. thuringiensis* are almost entirely due to the crystal toxin, often referred to as delta-endotoxin (Andrews et al., 1980; Fast, 1977). Andrews et al., (1987) have argued that the term "endotoxin" is inappropriate, because this term is commonly used to refer to toxic lipopolysaccharide components of cell walls from Gram-negative cells. (The toxin of *B. thuringiensis* shares some striking similarities to the

toxins of *Clostridium botulinum* and *Clostridium perfringens*; [Andrews and Bulla, 1981].) Spores alone are much less effective than either crystals alone or a mixture of the two. The relative ineffectiveness of spores alone is apparent in laboratory infectivity studies. Also, under field conditions, reinfection after application of insecticides is difficult to demonstrate (Couch and Ignoffo, 1981). In contrast, some evidence supports the view that other factors produced by *B. thuringiensis* are important in pathogenesis. As discussed in more detail below, β-exotoxin and certain proteases are produced by growing cells and are clearly active against insect systems. Some insects probably require viable spores for pathogenicity (Bulla et al., 1985). Finally, when an insect larva dies, the dead insect carcass usually contains relatively large quantities of spores and crystals (Aly, 1985; Aly et al., 1985; Prasertphon et al., 1974), indicating that septicemia typically follows the toxemia.

The observations that epizootics caused by *B. thuringiensis* occur relatively infrequently in nature, that the organism can be readily isolated from soils, that the crystal toxin is a normal component of the spore coat (Aronson et al., 1982; Somerville et al., 1968; Tyrell et al., 1981), and that *B. thuringiensis* is a relatively close relative of the common soil bacterium *B. cereus* (Andrews et al., 1987) imply a model for the microbe's ecology. This evidence suggests that the primary habitat of *B. thuringiensis* is soil. Somewhere over the course of evolution a common parent gained, either by mutation or genetic exchange, a spore coat protein that was toxic to insect larvae. By overproduction of this protein, the organism was able to kill an insect host and use the nutrients from that host in a saprophytic mode. This model would imply that the normal habitat for *B. thuringiensis* is soil, but that as the opportunity presents itself, the organism is able to use the insect as a temporary niche.

THE CRYSTAL TOXIN. As previously stated, *B. thuringiensis* is probably best known as a pathogen of insects. In nature, the disease is restricted to insect larvae; in the laboratory, toxicity to some adult insects of crystal protein has been reported when administered orally and/or rectally (Klowden and Bulla, 1984; Klowden et al., 1985). Moreover, the development stage of the larvae has a profound effect on its susceptibility to the toxin. Generally, younger larvae are more susceptible than older larvae (Andrews et al., 1987; Rasnitsyn et al., 1988). Because the route of entry of *B. thuringiensis* is through the oral cavity, ingestion of the toxin is mandatory, which requires insect feeding activity (Bulla et al., 1985).

Insects susceptible to the *B. thuringiensis* crystal toxin generally have alkaline midguts (pH ranges typically 10–12). At such pH the crystals solubilize and for some toxin types further activation may follow. Within the first minutes, a paralysis of the midgut and mouthparts ensues; these symptoms are accompanied by a drop in the pH of the insect gut and an increase in the hemolymph pH. The histological effects of the crystal toxin seem to be confined to the larval midgut epithelium and peritrophic membrane. Scanning electron micrographs of the larvae of *Manduca sexta* show that as soon as 1 h after oral administration of the toxin the microvilli in the midgut become shrunken, and after 4 h extensive midgut damage is observed. These histological changes are accompanied by a series of anomalies in the larvae's physiology. There is a severe restriction of potassium transport that is believed to be responsible for the maintenance of the high midgut pH. Active transport of potassium is inhibited about 78% within 10 min after ingestion, and, after several hours, all active transport ceases. Shortly after ingestion, the insect's peritrophic membrane becomes more permeable to particulate matter (Adang and Spence, 1983; Andrews et al., 1987; Gupta et al., 1985).

The mode of action of the crystal toxin has been reviewed (Andrews et al., 1987; Höfte and Whiteley, 1989). There is a marked similarity between the pathology of insects that have ingested the *B. thuringiensis* crystal toxin and that of humans suffering from cholera. Because of this similarity, much work has focused on the effect of the toxin on adenyl cyclase and intracellular cyclic AMP (cAMP) levels in diseased insects and in cultured cells that have been treated with toxin preparations. Knowles and Farndale (1988) found that treatment of cultured cabbage moth cells with *B. thuringiensis* toxin induced elevated cAMP levels and adenyl cyclase activity. However, when these cells were treated with the bee-venom toxin melittin, which also lyses the cells, they found similar effects. Therefore, the authors reasoned that the effect was secondary and was not directly related to the toxin's activity but rather consisted of a cellular response to lytic action.

Some evidence is beginning to accumulate relating to the mode of action of the toxin and its target or binding site. Knowles and Ellar (1987), for example, showed that insect cells cultured in the presence of ^{51}Cr, ^{86}Rb, and ^{3}H-uridine rapidly released those small molecules when treated with *B. thuringiensis* toxins that came from different subspecies. The receptor molecule may lie in the brush border membrane of epithelial cells. Sacchi et al. (1986) demonstrated that in midgut cells of the cabbage butterfly *Pieris brassicae,* K+-gradient-dependent

amino acid transport across the brush border membrane was inhibited by the *B. thuringiensis* toxin. Moreover, Hofmann et al. (1988a) demonstrated the presence of high affinity binding sites for the toxin of *B. thuringiensis* subsp. *thuringiensis* on the brush border membrane of midgut epithelial cells from *P. brassicae.* Several lines of evidence suggested that these were the natural binding sites for the *B. thuringiensis* toxin. I[125]-labelled toxin readily bound to the target cells, whereas toxin binding to nontarget cells (rat small intestine epithelial cells) was observed with much lower affinity (Hofmann et al, 1988a). I[127]-labelled, or unlabelled toxin competitively inhibited binding to the target cells but did not inhibit binding to the nontarget cells. The toxin from *B. thuringiensis* subsp. *thuringiensis,* although highly toxic for *P. brassicae,* is not toxic for larvae of the tobacco hornworm *Manduca sexta.* The toxin did not bind significantly to brush border membrane vesicles prepared from the larval midgut of *M. sexta,* a further indication that the binding to *P. brassicae* cells is specific (Hofmann et al., 1988b). Finally, Knowles et al. (1984) examined the effect of toxin on *Choristoneura fumiferana* CF1 cells in vitro. Preincubation of the toxin with N-acetylgalactosamine and N-acetylneuraminic acid specifically inhibited lysis of the target cells, and because N-acetylneuraminic acid has not been known to occur in insects, it was concluded that the toxin may recognize a specific plasma membrane glycoconjugate receptor with a terminal N-acetylgalactosamine residue. Considered togther these results suggest that the crystal toxin of many strains of *B. thuringiensis* binds to a glycoconjugate receptor on the brush border membrane of the insect midgut epithelium and then induces pore formation, resulting in leakage of the cells, followed by lysis.

OTHER PATHOGENIC FACTORS. Because of the importance of the crystal toxin to the activity of *Bacillus thuringiensis* insecticides, there has been a focus in research on this agent as a cause of insect pathology. It must be recognized, however, that other pathogenic factors are produced by certain *B. thuringiensis* isolates. Because of the nature of these factors, it is difficult to understand how they would function in ways other than to aid in an invasive propagation of *B. thuringiensis* cells in the insect.

One such factor is the so called β-exotoxin. The β-exotoxin, or fly factor, produced by some strains of *B. thuringiensis* is a heat stable nucleotide analog that is a potent inhibitor of RNA polymerase. This toxin is produced by β-exotoxin-positive cells during the stationary phase of growth and is broadly toxic to a variety of insects, both larvae and adults. Fly factor is an inhibitor of insect, mammalian, and bacterial RNA polymerases, and this activity is thought to be its mode of action. The heat stable properties and the toxic activity to *Musca domestica,* the house fly, are used to identify β-exotoxin activity in extracts (Beebee et al., 1972; Iandolo et al., 1976; Johnson, 1976, 1978; Johnson et al., 1975; Kim et al., 1972). Because of its toxicity and the possibility that it may be mutagenic, β-exotoxin-producing strains are currently banned in insecticidal formulations, but consideration has been given to the use of this factor itself as an insecticide (Bulla et al., 1985). As a final note, it is possible that the primary function of β-exotoxin is not as a pathogenic factor. Several kinds of observations lead to this conclusion. 1) β-Exotoxin is found primarily in spores, not in vegetative cells (Johnson, 1976) as would be expected for an invasive factor. 2) The RNA polymerases from sporulating cells of *B. thuringiensis* are up to 50% less sensitive to β-exotoxin than are those from vegetative cells, *E. coli*, or insects (Johnson, 1978). 3) Finally, there is no evidence to suggest that β-exotoxin-positive cultures are more pathogenic than are β-exotoxin-negative strains.

This evidence might lead to an alternative explanation for the presence of the toxic factor. Perhaps β-exotoxin is a normal regulatory factor important for control of sporulation in *B. thuringiensis*; the normal function might be to suppress expression of vegetative genes during sporulation. Under normal conditions, β-exotoxin is present at levels below normal detection in the so-called negative isolates. Strains that are termed positive simply have evolved a mechanism to overproduce the toxin, thus providing them a slight selective advantage in nature. Clearly, a better understanding of the role and occurrence of the β-exotoxin in *B. thuringiensis* is required to answer this question.

Many early investigators looked for specific immune systems analogous to the antibody response elicited by vertebrate animals when attacked by a bacterial invader. No specific agglutinating activity has been observed in insects. Insects do react with a defensive response during an intrusion by a bacterial invader, but the reaction is non-specific. The cecropins and attacins are two such classes of defensive proteins. These proteins, analogs of which have been identified in a wide variety of insects, are thought to function to lyse bacterial cells; the action is non-specific in that proteins induced by one bacterial species, for example, *E. coli* lyse cells from another species, *B. thuringiensis* (Hultmark et al., 1982, 1983; Hurlbert et al., 1985; Kaaya et al., 1987; Spies et al., 1986).

The finding that *B. thuringiensis* has been observed to produce a protease capable of spe-

cifically inactivating cecropins and attacins is particularly intriguing. This protein, inhibitor A, has been shown to attack and selectively destroy cecropins and attacins and thus to reduce the insect defense response. The protease is specific; although it does not seem to recognize a specific sequence, it attacks an open hydrophobic region near the C-terminus of the cecropin, and it does not attack globular proteins (Dalhammar and Steiner, 1984). The similarity between this pathogenic factor and an analogous factor produced by *Pseudomonas* species involved in infections in cystic fibrosis patients is striking. In the case of cystic fibrosis, it has been shown that several *Pseudomonas* isolates produce proteases that specifically degrade certain immunoglobins (Fick et al., 1984; Holder and Wheeler, 1984).

CRYSTAL TOXIN BIOCHEMISTRY. Despite the other pathogenic factors produced by *Bacillus thuringiensis*, by far the most research effort has been placed on the crystalline toxin, and this agent is therefore the best understood. There is a striking correlation between the shape of the parasporal crystal and the spectrum of toxicity it displays. The lepidopteran-toxic crystals are bipyrimidal in shape, the dipteran toxic crystals are pleomorphic, and coleopteran-toxic crystals are rectangular and flat. If toxicity truly resides in a discrete polypeptide subunit and if the individual polypeptide subunits have different ranges of toxicity to insects, then the molecular details of their structures must differ in significant ways and, hence, their crystals should have different topographical shapes. Therefore, it is possible that significant information about specificity of the proteins produced by a given isolate may be derived from a study of the crystal configuration.

Immunological comparison of the crystal toxin antigens has already been discussed. Antisera against crystals from several strains of pathotype I and from subsp. *israelensis* (pathotype II) were prepared by Tyrell et al. (1981). In Ouchterlony double diffusion assays, cross-reacting epitopes were not observed between pathotype I strains and the pathotype II strain, whereas an evidently homologous reaction was observed among the pathotype I strains. Using the more sensitive ELISA technique, however, differences among the pathotype I strains were noted and some similarity between these and the subsp. *israelensis* crystal proteins was apparent (Wie et al., 1982). Interestingly, crystal proteins from subsp. *tenebrionis* (pathotype III) did not serological cross-react with proteins from crystals of the other two pathotypes (Krieg et al., 1987b).

The crystals from all three pathotypes share some common properties. The most obvious of these is that they are all protein. Moreover, despite the antigenic diversity, the polypeptides contained in the crystals tend to have some common size ranges. For example, when crystal proteins from pathotypes I and II are separated on sodium dodecyl sulfate (SDS) polyacrylamide gels, major bands appear in the molecular weight (mol wt) range of 120,000–140,000 and a second band or group of bands in the mol wt range of 60,000–70,000. In addition, pathotype II crystals contain a third polypeptide (mol wt range of 23,000–30,000). Pathotype III crystals contain only proteins in the middle range. Under conditions of neutral and acid pH, the crystals are insoluble; in fact, the insolubility of the proteins and their tendency to re-form crystals and precipitate under such conditions remains a major problem in the laboratory. As previously discussed, insects sensitive to the crystal toxin of *B. thuringiensis* share the common property of having alkaline midguts. Not surprisingly, the crystals from all three pathotypes become soluble under alkaline conditions and in all cases the proteins retain toxicity. The crystal proteins from subsp. *israelensis* lose a substantial portion of their toxicity in insect bioassays when they are solubilized. However, this has been shown to result from a problem in the insect assay procedure rather than from damage to the protein itself, because when the solubilized crystal proteins are adsorbed onto latex beads, the proteins regain most of their toxicity (Andrews et al., 1981; Bulla et al., 1981; Calabrese et al., 1980; Huber et al., 1981; Insell and Fitz-James, 1985; Lilley et al., 1980; Schnell et al., 1984; Tyrell et al., 1979).

PATHOTYPE I CRYSTAL PROTEINS. Crystal proteins from pathotype I isolates share a number of common features. The most obvious of these has already been pointed out; namely, they usually share a common crystal shape and contain proteins in a common size range (130,000–140,000). They are antigenically cross-reactive. The protein subunits are protoxin molecules that are converted to a toxic form after ingestion by a susceptible insect. Upon ingestion, the crystal becomes soluble in the insect midgut and is then activated, via proteolytic cleavage, to a toxin (mol wt = 68,000). The evidence suggests that the proteases responsible for toxin activation are insect-derived. Crystal proteins from subsp. *kurstaki* strain HD1, for example, are composed of repeating subunits of mol wt = 135,000 (Bulla et al., 1981). When the crystals are solubilized at pH 12, dialyzed against a pH 7.5 buffer, and allowed to stand at room temperature for several days, there is a conversion of some of the material to a smaller molecule (mol wt = 68,000) that is thought to be the toxin (Bulla et al., 1979). When these data appeared in the literature there were several items of controversy regarding this

model. One area of concern was that the yield of toxic product was low relative to the quantity of starting crystal protein used, leading to some speculation that this was an artifact. Andrews et al. (1985) showed that under appropriate conditions the conversion of protoxin to toxin could be done in vitro using commercially available trypsin and that this treatment did not reduce the toxicity significantly. In these studies the soluble proteins from subsp. *kurstaki* were approximately four fold more toxic than were whole crystals. When these proteins were dialyzed against a dilute carbonate buffer and treated with limiting quantities of trypsin, nearly all the toxic activity (>90%) in the soluble crystal preparation was recovered, but the protein in the solution was now almost exclusively of a mol wt = 68,000. This means that the molar toxicity of the preparation remained constant, whereas the LC_{50} (concentration that is lethal to 50% of the treated insects) of the proteins had approximately doubled. Similar conversions were demonstrated using insect gut-derived proteases, confirming that enzymes from the larval midgut probably were responsible for the conversion in vivo (Tojo and Aizawa, 1983).

These data left one observation unexplained; crystals purified from all pathotype I strains examined before 1985 contained a molecule of approximately 68,000 mol wt (Tyrell et al., 1981). Since purified toxin comigrated with this smaller molecule, there was controversy over its origin. A better understanding of this problem came with the recognition that a strain of subsp. *kurstaki* (HD251) evidently did not contain, or contained very little, of the 68,000 mol wt protein in its crystals. Upon further examination, strain HD251 was shown to produce greatly reduced levels of intracellular proteases, but produced normal levels of toxicity. Subsequently, it was shown that the small quantity of 68,000 mol wt protein found in crystals of most pathotype I strains probably resulted from conversion of protoxin to toxin by intracellular proteases produced by *Bacillus thuringiensis* during the sporulation process (Andrews et al., 1985; Bibilos and Andrews, 1988).

Genetic observations confirmed conclusions derived from protein chemistry. From 1981 to 1989 at least 49 reports of cloning and/or sequencing of *B. thuringiensis* crystal toxin genes appeared in the literature (Table 1), and 30 of these reports were of genes encoding proteins with toxicity to Lepidoptera. Only two of these reports were of genes encoding proteins in the size range of 60,000–70,000 mol wt, whereas all of the others were of genes encoding proteins in the size range of 120,000–140,000 mol wt. Moreover, because both of the smaller proteins show a toxicity to both lepidopteran and dipteran larvae, it is unclear that these are true pathotype I toxins. Therefore, the DNA cloning and sequence data confirm that the crystal toxin subunit in pathotype I crystals are usually, if not always, composed of proteins of mol wt 120,000–140,000. DNA sequencing studies also confirmed the protoxin to toxin conversion hypothesis. Data from a number of laboratories showed that the toxin molecule was derived from the amino-terminal portion of the protoxin (Schnepf et al., 1985; Wabiko et al., 1985).

Crystals from some isolates contain multiple toxin proteins, each with a distinct host range of toxicity, and the total activity spectrum of the crystal represents a summation of the individual toxic spectra. For example, the crystals from subsp. *aizawai* contain at least two different toxin proteins, one of which is specific in its activity for *Pieris brassicae* and the other for *Spodoptera littoralis,* both of which are lepidopterans (Lecadet et al., 1988). The two types of subsp. *aizawai* genes have been cloned and their distinct toxicities confirmed (Sanchis et al., 1988). Similar data were provided by Knowles and Ellar (1988), who observed that subsp. *aizawai* contained two proteins, one of which was toxic to *Choristoneura fumiferana* and the other to *S. frugiperda.* Multiple genes have been cloned from subsp. *entomocidus* (Visser et al., 1988), subsp. *morrisoni* (Granum et al., 1988), subsp. *thuringiensis* (Brizzard and Whiteley, 1988), and subsp. *kurstaki* (Widner and Whiteley, 1989).

There may be some clues in the literature regarding the significance of these different genes. In a report that is both interesting and alarming, McGaughey (1985) observed resistance to the *B. thuringiensis* toxin in an insect population. While trying to use *B. thuringiensis* to control *Plodia interpunctella* in grain bins, he observed that in some cases the populations were suppressed, whereas in others a similar concentration of insecticide did not achieve effective results. Upon further examination, it was observed that in as few as 15 generations of continual selection, the LC_{50} of the toxin increased nearly 100-fold. That a population can become resistant in such a short time raises obvious concerns about the use of *B. thuringiensis* insecticides. Interestingly, however, in later studies it was noted that insects were selectively resistant to certain toxin types while still sensitive to others (McGaughey and Johnson, 1987). The presence of resistance in a population may explain the presence of multiple toxin genes within a single isolate of *B. thuringiensis;* multiple genes may be a mechanism for overcoming the development of resistance. At the present time, resistance has only been observed in *P. interpunctella,* and it is unclear how widespread or universal this resistance will become.

Table 1. Survey of reported *B. thuringiensis* crystal protein genes cloned between 1981 and 1989. Adapted from Hurley (1989).

Source of the cloned gene (subspecies and/or strain)	Type of report (cloning and/or sequence)	Insect specificity of gene	Molecular weight of protein coded by gene	Reference
kurstaki HD1	Cloning	Lepidoptera	133,000	Schnepf and Whiteley, 1981
kurstaki HD1	Cloning	Lepidoptera	134,000	Held et al., 1982
thuringiensis 1715	Cloning	Lepidoptera	130,000	Klier et al., 1982
kurstaki HD73	Cloning	Lepidoptera	133,000	Kronstad and Whiteley, 1984
israelensis	Cloning	Cytolysin	26,000	Ward et al., 1984
kurstaki HD1	Cloning	Lepidoptera	133,000	Schnepf et al., 1985
kurstaki HD73	Cloning and sequence	Lepidoptera	133,000	Adang et al., 1985
aizawai	Cloning	Lepidoptera	133,000	Klier et al., 1985
kurstaki HD244	Cloning	Lepidoptera	140,000	McLinden et al., 1985
israelensis	Cloning	Diptera	Not reported	Sekar and Carlton, 1985
sotto	Cloning and sequence	Lepidoptera	144,000	Shibano et al., 1985
isrealensis	Cloning and sequence	Cytolysin	28,000	Waalwijk et al., 1985
thuringiensis 1715	Cloning	Lepidoptera	140,000	Wabiko et al., 1985
israelensis	Cloning	Cytolysin	28,000	Bourgouin et al., 1986
		Diptera	130,000	
kurstaki HD1	Cloning and sequence	Lepidoptera	130,000	Geiser et al., 1986
san diego	Cloning	Coleoptera	65,000	Herrnstadt et al., 1986
thuringiensis	Cloning and sequence	Lepidoptera	130,000	Hofte et al., 1986
thuringiensis	Cloning	Lepidoptera	120,000	Honigman et al., 1986
kurstaki HD1	Cloning	Lepidoptera	130,000	Kronstad and Whiteley, 1986
thuringiensis HD2	Cloning	Lepidoptera	130,000	Kronstad and Whiteley, 1986
kurstaki	Cloning	Lepidoptera	135,000	Shivakumar et al., 1986
kurstaki	Cloning and sequence	Lepidoptera	131,000	Thorne et al., 1986
israelensis	Cloning and sequence	Diptera	58,000	Thorne et al., 1986
thuringiensis	Sequence	Lepidoptera	130,000	Wabiko et al., 1986
israelensis	Cloning	Diptera	130,000	Angsuthanasombat et al., 1987
aizawai HD133	Cloning	Lepidoptera	135,000	Chak and Ellar, 1987
kurstaki HD1	Sequence	Lepidoptera	135,000	Fischhoff et al., 1987
morrisoni	Cloning and sequence	Cytolysin	27,000	Galjart et al., 1987
aizawai IC1	Cloning	Lepidoptera and Diptera	130,000	Haider et al., 1987
san diego	Sequence	Coleoptera	65,000	Herrnstadt et al., 1987
tenebrionis	Cloning and sequence	Coleoptera	72,000	Hofte et al., 1987
aizawai IPL7	Cloning and sequence	Lepidoptera	131,000	Oeda et al., 1987
tenebrionis	Cloning and sequence	Coleoptera	72,000	Sekar et al., 1987
israelensis	Cloning and sequence	Diptera	130,000	Ward and Ellar, 1987
thuringiensis HD2	Cloning and sequence	Lepidoptera	130,000	Brizzard and Whiteley, 1988
israelensis	Sequence	Diptera	130,000	Chungiatupornchai et al., 1988
israelensis	Cloning and sequence	Cytolysin	28,000	Donovan et al., 1988a
		Diptera	72,000	
kurstaki HD263	Cloning and sequence	Diptera and Lepidoptera	66,000	Donovan et al., 1988b
morrisoni HD12	Cloning	Diptera and Lepidoptera	140,000	Granum et al., 1988
tenebrionis	Cloning and sequence	Coleoptera	73,000	McPherson et al., 1988
aizawal 7.29	Cloning	Lepidoptera	130,000	Sanchis et al., 1988
entomocidus	Cloning	Lepidoptera	130,000	Sanchis et al., 1989
israelensis	Cloning and sequence	Diptera	130,000	Sen et al., 1988
		Diptera	130,000	
israelensis	Sequence	Diptera	128,000	Tungpradubkul et al., 1988
entomocidus	Cloning	Lepidoptera	Not reported	Visser et al., 1988
israelensis	Cloning	Diptera	130,000	Ward and Ellar, 1988
		Diptera	130,000	
gulleriae	Cloning	Diptera and Lepidoptera	61,000	Ahmad et al., 1989
entomocidus	Cloning and sequence	Lepidoptera	Not reported	Masson et al., 1989
		Lepidoptera		
kurstaki	Cloning and sequence	Lepidoptera	130,000	Widner and Whiteley, 1989
		Lepidoptera	130,000	

Adapted from Hurley (1989).

PATHOTYPE II CRYSTAL PROTEINS. As previously indicated, one unique feature of the pathotype II isolates is that their crystals contain a polypeptide smaller than that observed in the other two pathotypes. Moreover, this smaller protein (mol wt = 23,000) is clearly the most abundant of the proteins in the crystal toxin. Also, the various polypeptides found in crystals of subsp. *israelensis* did not antigenically cross-react with each other, unlike the situation with pathotype I strains. Specifically, the proteins in the 120,000–140,000 mol wt range react with antibody prepared against the proteins in the 60,000–70,000 mol wt range, but the 23,000 mol wt proteins do not; nor do the larger proteins react with antibody prepared against the 23,000 mol wt protein (Pfannenstiel et al., 1986; Tyrell et al., 1981).

Shortly after the initial observations regarding the polypeptide profiles in subsp. *israelensis,* a potent and highly nonspecific cytolytic activity was observed in these crystals. A number of investigators quickly purified the activity and ascribed it to the 23,000 mol wt protein (Armstrong et al., 1985; Chilcott and Ellar, 1988; Davidson and Yamamoto, 1984; Ibara and Federici, 1986; Insell and Fitz-James, 1985). Many of these investigators observed both cytolytic and toxic activity associated with this protein and identified it as the toxin molecule. The protein is active on cells from insects of several orders, on mammalian cells, and even on some bacterial cells. From a functional standpoint it was difficult to understand how such a nonspecific activity when tested on cells in vitro could have such a specific effect in vivo. Moreover, the toxicity of the purified cytolysin was approximately 100- to 1000-fold less than that of solubilized proteins of whole crystals. Accordingly, Hurley et al. (1985) first demonstrated that the activities could be separated, and then purified the 23,000 and 68,000 mol wt polypeptides (Hurley et al., 1987). The smaller protein, when purified, was highly cytolytic, whereas the larger protein was toxic to mosquito larvae (1000 times more toxic than the cytolysin). In addition to observing toxic activity in the 68,000 mol wt protein, high levels of toxicity were observed in the larger proteins as well. Hurley et al. (1987) attributed the disagreement between their data and that of the others to two factors: 1) In one report, a 65,000 mol wt molecule was purified and shown to be relatively nontoxic (Ibarra and Federici, 1986). However, these investigators used SDS to solubilize the toxin preparation; and at least with the lepidopteran toxins, this had previously been shown to eliminate activity (Huber et al., 1981). 2) When in purified form, the toxin was unstable and readily degraded to smaller nontoxic forms. It was unclear whether this instability was intrinsic or caused by contam-

inating protease. Proteases associated with the crystal toxin of subsp. *israelensis* had already been reported (Chilcott et al., 1983). Although evidence from protein chemistry was becoming convincing, the ultimate proof would come from gene cloning experiments.

Bourgouin et al. (1986) constructed a library of *B. thuringiensis* subsp. *israelensis* plasmid DNA in an *E. coli* plasmid vector and identified three genes coding for proteins of 26, 73, and 135 kDa, respectively, located on a single 75-kb plasmid. Interestingly, the 73 and 135 kDa proteins produced from the cloned genes were both toxic, and the 73-kDa protein appeared to be a truncated version of the larger gene. The 23-kDa protein-encoding gene did not appear related to the other two, and the protein produced from the cloned gene was not toxic but was highly cytolytic. The surveyed gene cloning literature seems to support their conclusions. Of the 49 reports of cloned and/or sequenced crystal toxin genes from *B. thuringiensis* in the literature, five are of cytolysin genes, and all five genes encode proteins having molecular weights in the range of 26,000–28,000 (Table 1). There are 16 reports of dipteran toxic protein genes (including lepidopteran and dipteran toxic protein genes) from *B. thuringiensis* in the surveyed literature and of these four genes code for proteins in the 60–70 kDa range, whereas 11 code for proteins in the 120–140 kDa range, and 1 has no reported molecular weight (Table 1).

Since the isolation of subsp. *israelensis* there have been other isolations of pathotype II *B. thuringiensis* strains reported. These evidently belong to other subspecies based on their serotype and probably have proteins in their crystals similar to those in subsp. *israelensis* (Gill et al., 1987; Padua et al., 1980). Although the cytolysin is not the primary toxin, it is not without function. Evidence suggests that the cytolysin may act synergistically with the toxin (Hurley et al., 1987; Wu and Chang, 1985). There are evidently two forms of the toxin gene; however, their role is not understood.

PATHOTYPE III CRYSTAL PROTEINS. Owing to the relatively recent isolation of pathotype III strains, there is far less information regarding these crystal toxin proteins. It is interesting to note, however, that most of the controversy regarding the crystal proteins from the other two pathotypes was resolved not by protein chemistry but by gene cloning technology. Because pathotype III strains were isolated after gene cloning technology became available, it is not surprising that there has been much less controversy regarding the content of these crystals. The prototype strain for pathotype III is subsp. *tenebrionis.* The crystals from subsp. *tenebrionis*

appear to contain two polypeptides of mol wt 68,000 and 72,000 (Krieg et al., 1987b). Five of the 49 cloned and/or sequenced genes reported in Table 1 are for pathotype III crystal proteins, and the data are in remarkable agreement with the protein chemistry data. The literature regarding pathotype III strains is not without controversy, however. There has been a report of a second subspecies of pathotype III, termed subsp. *san diego* (Ferro and Gelernter, 1989; Herrnstadt et al., 1986) but there are claims that this strain is identical to subsp. *tenebrionis* (Krieg et al., 1987a).

CRYSTAL TOXIN SYNTHESIS. There is substantial literature to support, at least with most subspecies of *B. thuringiensis*, that the production of crystal toxin is linked to sporulation. Several lines of evidence support this conclusion. First, the parasporal crystals appear in the cells in close proximity to the spore, and the time of their appearance when viewed by phase contrast and electron microscopy closely coincides with spore formation (Bechtel and Bulla, 1976). Fig. 1 shows a diagram of the relationship between sporulation and crystal toxin production in subsp. *kurstaki*. Moreover, crystal toxin antigen accumulates in the cells only between 4 and 6 h after the onset of sporulation (Andrews et al., 1981).

The synthesis of crystal toxin is evidently controlled at the level of transcription. Synthesis of mRNA specific for crystal toxin, as measured by in vitro translation (Andrews et al., 1982) and Northern blotting (Kronstad and Whiteley, 1984), correlates with the accumulation of crystal toxin antigen in sporulating cells. Moreover, there is evidence that formation of mRNA specific for crystal toxin requires a unique form of RNA polymerase (Brown and Whiteley, 1988). In subsp. *israelensis* the presence of a 27-kDa protein is required (Adams et al., 1989). There is a notable exception to the association between crystal toxin production and sporulation, however. In subsp. *tenebrionis,* it appears that crystal toxin proteins are synthesized in vegetative cells (Sekar, 1988).

Genetics

The most extensive studies on the genetics of *B. thuringiensis* have focused on cloning and characterization of the crystal toxin genes. This information was presented in the previous section to aid in understanding the nature of the toxins and will not be reiterated in this section.

PLASMID BIOLOGY. *B. thuringiensis* isolates typically contain numerous plasmids, ranging in size from 2 to >200 kb. A given strain may carry up to 17 plasmids of distinct sizes (Gonzalez et al.,
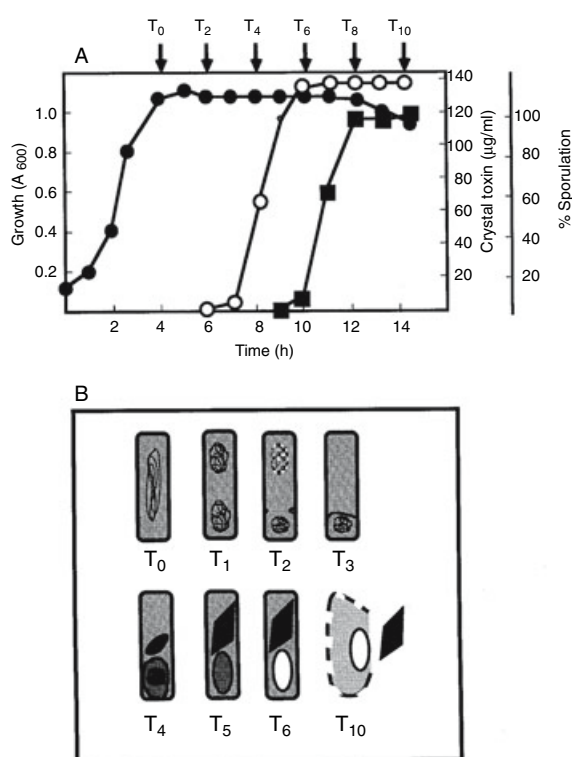
Fig. 1. Growth, sporulation, and crystal toxin formation in *B. thuringiensis*. (A) A typical growth curve of *B. thuringiensis* subsp. *kurstaki*. The solid circles represent growth, the open circles show the formation of crystal toxin antigen, and the closed squares show the percentage of cells that contain complete, phase-light spores. (B) An artist's conception of growth, sporulation, and crystal toxin production in *B. thuringiensis* subsp. *kurstaki*. The data are based on electron micrographs published by Bechtel and Bulla (1975) and on phase-contrast microscopic examination of the sporulating cultures shown in A. The cells were grown in liquid GYS medium as described in the text. Crystal toxin was determined as described by Andrews et al. (1981).

1981; Lereclus et al., 1982; Stahly et al., 1978). Several aspects of *B. thuringiensis* plasmid biology are unique and worthy of mention in this context.

In some strains with many plasmids (some of which are very large), up to 20% of the total DNA coding capacity may be plasmid-borne. Most of the plasmids in *B. thuringiensis* are cryptic in that functions have not been assigned to them; indeed, most of the plasmids from these strains can be cured with no observable change in phenotype (Gonzalez et al., 1981). It is unlikely, however, that an organism would maintain this amount of unproductive DNA, so it is likely that these cryptic plasmids serve some purpose.

Plasmid incompatibility is the inability for two plasmids to coexist in the absence of selective

pressure. When two plasmids are of the same incompatibility group, they will not be maintained unless each contains a selective marker that can be used to assure that it is not lost. Because of the multiple plasmids found in *B. thuringiensis* isolates and because plasmid loss is not readily observed in these strains without some curing pressure, one must conclude that 1) there must be many incompatibility groups (at least 17) in *B. thuringiensis*; 2) that in cases where two or more of the plasmids are of the same incompatibility group, there must be some as of yet unrecognized selective pressure to maintain these plasmids; and/or 3) *B. thuringiensis* contains some mechanisms to defeat normal incompatibility groupings. In any case, further investigation of the multiple plasmids contained in *B. thuringiensis* should increase our understanding of plasmid incompatibility in bacteria in general.

Finally, *B. thuringiensis* plasmids have been shown to be transferred with relatively high frequency between many bacilli, including *B. thuringiensis*, *B. cereus*, *B. megaterium*, and *B. anthracis* (Battisti et al., 1985; Gonzalez et al., 1982; Green et al., 1989). Further understanding of this process will greatly extend the understanding of genetic exchange in Gram-positive bacteria in general, and further define safety parameters for genetically engineered microbes released into the environment.

Transposons and Insertion Sequences. Lereclus et al. (1983) described a unique 4.2-kb DNA sequence that was found on several large toxin-coding plasmids of *B. thuringiensis*. This particular sequence (Th-sequence) was found as an insertion in the *Streptococcus faecalis* plasmid, pAMβ1, which was introduced into *B. thuringiensis* subsp. *berliner* 1715. The 4.2-kb DNA sequence resembled an insertion sequence (IS element) and was found in close proximity to the protoxin genes from several *B. thuringiensis* subspecies. A 1.3-MDa segment of DNA separated the Th-sequence from the subsp. *berliner* 1715 protoxin gene, and a similar DNA segment was located on the other extremity of the gene but in the opposite orientation. These DNA segments contained inverted repeat sequences and were referred to as IR*1*. When a plasmid carrying IR*1* and the Th-sequence was observed with an electron microscope, it appeared to be a transposon (Tn) (Lereclus et al., 1984). Therefore, the Th-sequence is now referred to as Tn*4430,* and is the first transposon isolated from the genus *Bacillus* (Lereclus et al., 1986).

This DNA segment is similar to the Tn3 family in structure and transpositional activity (Lereclus et al., 1986). In addition to its insertion properties, Tn*4430* has short terminal inverted repeats and promotes deletions adjacent to its insertion site similar to that observed with Class II elements (Lereclus et al., 1984, 1986). Nonetheless, the function of this transposon has not been identified. Similar inverted sequences, IR*1750* and IR*2150,* flanking the plasmid-encoded 68-kDa protoxin gene of subsp. *kurstaki* HD-73, have been identified (Kronstad and Whiteley, 1984). Southern hybridization confirmed that these sequences were located in close proximity to the protoxin genes from 14 other strains of *B. thuringiensis*, and it was believed that IR*1750* and IR*2150* may be insertion sequences that mediate transposition. Nucleotide sequencing of one IR*1* element revealed all the characteristic features of an insertion sequence and was referred to as IS*231* (Mahillon et al., 1985). IS*231* (1656 bp) was isolated from subsp. *berliner* 1715 and found to be closely linked to the protoxin gene. It is delineated by two 20-bp inverted repeats flanked by two 11-bp direct repeats. IS*231* contains an open reading frame that spans almost the entire sequence, and DNA sequence homology has been observed between IS*231* and the *E. coli* IS*4* element.

Mahillon et al. (1987) sequenced two other insertion regions flanking the subsp. *berliner* 1715 crystal toxin gene and showed them to be variants of IS*231*. These sequences were referred to as iso-*231* elements. When the nucleotide sequences surrounding the iso-*231* elements were compared to IS*231,* it was shown that a structural association existed between these elements and the transposon Tn*4430.* It appeared that two IS*231* elements transposed into Tn*4430* where both the IS*231*s and the transposon Tn*4430* remained structurally intact. This structural association between IS*231* and Tn*4430* is similar to the organization of class I mobile genetic elements (i.e., Tn*5* or Tn*10*), and although the exact function of the IS*231* elements is unknown, it is thought they control Tn*4430* transposition (Mahillon et al., 1987).

In summary, the presence of repeated elements around crystal protein genes may provide two possible mechanisms for the dispersal of the toxin gene on different plasmids and for the integration of this gene into chromosomal DNA. The evidence for transposition of the Th-sequence into other plasmids and the arrangement of the repeated elements suggest that the toxin gene could undergo transposition. Alternatively, the presence of repeated elements on many different plasmids and on chromosomal DNA would also provide a mechanism for rearrangements of DNA. Further research is required to understand transposition in *B. thuringiensis* and to provide an increased understanding of the role of these repeated elements.

GENETIC TRANSFER SYSTEMS. Although there is substantial literature regarding the genetics of *Bacillus thuringiensis*, methodology for the introduction of foreign or cloned DNA into this organism is minimal. Three general mechanisms for introduction of DNA are usually described, transformation, conjugation, and transduction. All three forms of genetic exchange involving *B. thuringiensis* have been described in the literature.

Several investigators have reported transformation of *B. thuringiensis* protoplasts using methods similar to those used in *B. subtilis* (Chang and Cohen, 1979). Martin et al. (1981), for example, used a protoplast transformation protocol to transform *B. thuringiensis* with plasmid pC194. Unfortunately, they were unable to detect extrachromosomal pC194 in regenerated transformants and provided evidence that the plasmid integrated into the chromosome. Later, other workers physically identified the stable presence of pC194 in various transformants (Crawford et al., 1987; Fischer et al., 1984; Heierson et al., 1987). Moreover, other plasmids have been inserted into cells of *B. thuringiensis* by protoplast transformation (Alikhanian et al., 1981; Miteva et al., 1981). The methods used by different investigators for protoplast generation has varied. Fischer et al. (1984) used protoplasts generated by lysozyme treatment, whereas Heierson et al. (1987) and Crawford et al. (1987) generated protoplasts by using conditions that induced limited autolysin formation.

High voltage electroporation has been used in many systems, including plant cells, animal cells, and bacteria for introduction of foreign DNA. Recently, Bone and Ellar (1989) demonstrated use of this technique to transform several plasmids into *B. thuringiensis*. The method is rapid and effective, and yields transformation efficiencies in the range of 10^2–10^5/µg of DNA.

The naturally occurring conjugation systems in *B. thuringiensis* have already been discussed. Gonzalez et al. (1982), for example, described a natural plasmid transfer system that allowed them to more extensively analyze the relationship between crystal production and plasmid content. They noticed high frequency transfer of plasmids during logarithmic growth between parasporal crystal-positive (cry^+) strains and cry^- strains. For instance, when *B. thuringiensis* subsp. *kurstaki cry$^-$* recipients were mated with cry^+ *B. thuringiensis* subsp. *thuringiensis* donors, the *B. thuringiensis* subsp. *kurstaki* strains were converted to cry^+ phenotype. The size of the transmissible crystal-coding plasmid varied with the donor strain, and immunological analysis showed the *B. thuringiensis* subsp. *kurstaki cry+* transcipients to be hybrid strains, i.e., having the flagellar serotype of the recipient and crystals of the donor serotype.

Gonzalez and Carlton (1984), utilizing their high-frequency plasmid transfer system, genetically analyzed crystal toxin production in *B. thuringiensis* subsp. *israelensis*. They showed that *B. thuringiensis* subsp. *israelensis* contained eight plasmids ranging in size from 3.3–135 MDa. Curing studies showed that a 75-Mda plasmid was responsible for toxin production, because 15 B. thuringiensis subsp. *israelensis cry$^-$* isolates were shown to lack this 75-MDa plasmid. This conclusion was supported by transfer of the 75-MDa plasmid into a cry^- strain.

Transfer systems native to other bacteria can also be used for introduction of genetic information into *B. thuringiensis*. Franke and Clewell (1981) described a chromosome-borne tetracycline resistance element in *Streptococcus faecalis* strain DS16 that had the characteristics of a transposon. This 15–17 kb element, Tn*916,* was capable of transposition to several different conjugative plasmids containing hemolysin genes (pAD1 and pOB1) at frequencies of approximately 10^{-6}. Moreover, Tn*916* was also capable of transposition at low frequency (10^{-8} per recipient) from plasmid-free derivatives of strain DS16 to plasmid-free *S. faecalis* recipients. This transfer was resistant to the action of DNase, did not require homologous host-mediated recombination functions, and occurred by a conjugation-like event that required direct contact between the donor and recipient. Tn*916* (16.4 kb) is classified as a Class IV transposon (Kleckner, 1981). Tn*916* has been transferred to a number of streptococcal species by the filter mating technique (Franke and Clewell, 1981; Nida and Cleary, 1983; Wanger and Dunny, 1985; Weiser and Rubens, 1987). Furthermore, Tn*916* has been transferred by filter matings to *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus anthracis*, *Clostridium difficile*, and *Mycoplasma hominis* (Hachler et al., 1987; Ivins et al., 1987; Jones et al., 1987; Kathariou et al., 1987; Roberts and Kenny, 1987). The conjugative transfer of Tn*916* in the absence of plasmid DNA occurs at a frequency of 10^{-5}–10^{-8} per donor, and DNA hybridization data has demonstrated that Tn*916* inserts into different sites on the recipient chromosome (Gawron-Burke and Clewell, 1982). Insertional mutagenesis using Tn*916* has been reported (Ivins et al., 1987; Kathariou et al., 1987; Nida and Cleary, 1983).

Naglich and Andrews (1988a) recently reported introduction of the *S. faecalis* transposon Tn*916* into *B. thuringiensis*. When introduced into *B. thuringiensis* by filter mating with *S. faecalis*, Tn*916* inserts into evidently random sites on the chromosome, as judged by Southern analysis; insertion into plasmid DNA has not been

observed. In contrast, when Tn*916* was transferred from one strain of *B. thuringiensis* subsp. *israelensis* to an antibiotic-resistant derivative of the same strain, insertion occurred at one site, suggesting that in this case DNA probably integrated into the chromosomal DNA by homologous recombination.

To enhance the transfer frequency, Tn*916* was first introduced into *B. subtilis* and then transferred to an antibiotic-resistant strain of *B. thuringiensis* subsp. *israelensis*. With this method, transfer occurred at frequencies 10– to 100-fold greater than when transfer was from *S. faecalis*. As in the case of transfer from *S. faecalis*, Tn*916* inserted randomly into the chromosome of *B. thuringiensis*, but, as in the previous experiments, not into plasmid DNA (Naglich and Andrews, 1988b). Several lines of evidence support the conclusion that DNA transfer is a Tn*916*-dependent conjugal event. 1) Transfer of genetic material was only observed when the cells were impinged on a filter and then grown on solid medium; transfer was not observed in broth. 2) Incorporation of high levels of DNase into the medium upon which the filter was placed did not prevent the process. 3) Transfer was Tn*916*-dependent; no DNA transfer was observed in the absence of the transposon, which carries its own mobilization functions.

The *Staphylococcus aureus* plasmids, pC194 and pUB110, were introduced into *B. thuringiensis* subsp. *israelensis* by using Tn*916* as a mobilizing agent. Transfer of pC194 occurred only when *B. thuringiensis* subsp. *israelensis* was mated with a *B. subtilis* donor that contained both pC194 and Tn*916;* plasmid transfer was not observed in the absence of the transposon. *B. thuringiensis* transconjugants resistant to chloramphenicol (the plasmid marker, Cm^r) and tetracycline (the transposon marker, Tet^r) were detected at a frequency of 2×10^{-6} per recipient cell, whereas the Tet^r phenotype but not the Cm^r phenotype was observed at a frequency of 1×10^{-4}. The converse, Cm^r but not Tet^r, was observed at a frequency of 3×10^{-5}. Transconjugants were detected in filter matings only, not in broth. Southern hybridization data indicated that the transposon was integrated into the *B. thuringiensis* chromosome, whereas the pC194 probe hybridized to a covalently closed circle form of the same size as pC194. The Tet^r phenotype was maintained during serial passage of *B. thuringiensis* without selection, whereas the Cm^r phenotype was not. The plasmid pUB110, also from *S. aureus*, can be transferred at similar frequencies, but mobilization of the plasmid pE194 has not been detected (Naglich and Andrews, 1988b).

Transduction in *B. thuringiensis* has also been reported. Lecadet et al. (1980), for example, demonstrated transfer of genetic markers in *B.* *thuringiensis* using the transducing phage CP54. A similar phage, CP51, has been shown to mediate generalized transduction in *B. thuringiensis*, as well as in *B. cereus* and *B. anthracis* (Thorne, 1978). CP51 transduces chromosomal markers at frequencies ranging from 10^{-6}–10^{-7} and can carry up to 60 kb of DNA (Yelton and Thorne, 1971). Also, phage CP51 induces movement of plasmid-borne DNA into *B. thuringiensis* (Ruhfel et al., 1984).

Biotechnological Applications

Despite the relatively large number of insect pathogens that have been proposed as insecticides, those products formulated with *B. thuringiensis* are the most extensively and widely used today. There have been recent reviews discussing the biotechnology of *B. thuringiensis* (Andrews et al., 1987; Bulla et al., 1985). Accordingly, only a few selected topics will be discussed herein.

Insecticides formulated with *B. thuringiensis* are extremely specific; they affect only a limited range of insects and are nontoxic to predatory insects, birds, and mammals, thus providing significant advantages over chemical insecticides. Because of their lack of toxicity to humans, these insecticides can be applied just prior to harvest, a time when an insect infestation can cause extreme damage to the crop in a short time. Because no human toxicity has been reported, there can be immediate field entry. Moreover, there is minimal risk of toxicity to those who apply the pesticide, and there is no significant risk to humans living near the application area. The latter is particularly important when mosquitoes must be controlled near residential areas.

There are, however, significant problems associated with the use of these insecticides. One is that they exhibit poor stability under field conditions. The insecticidal activity of pesticides formulated with *B. thuringiensis* has a half-life of 1–2 days (Beegle et al., 1981; Sorenson and Falcon, 1980). Therefore, frequent reapplications are required. At least in part because of this short field life, such insecticides are expensive to use when compared to the more widely used organic pesticides. Insecticidal activity is associated primarily with the crystal toxin rather than the spores (West et al., 1984). In a soil environment, the low stability of crystal toxin is probably due to degradation by soil microflora (West, 1984). The lack of the toxin's stability on foliage is due at least in part to physical removal by precipitation. Research is needed to discover methods for stabilizing the crystal toxin.

Bulla et al., (1985) discussed three possible reasons that *B. thuringiensis* insecticides have not been more widely used. 1) For many insect control situations, broader-spectrum insecticides

are preferred because of the multiple pests found on crops. Moreover, growers often believe that contact pesticides are more effective and have found that more cost-effective alternatives are available. Thus the marketplace is generally viewed as too small to attract widespread interest among agricultural companies. 2) Many agricultural companies are adapted to working with chemical pesticides and have difficulty working with a product of biological origin. 3) There has been little or no patent protection available to producers. The impact of the Chakrabarty court decision that allows patenting of genetically modified microbes remains unclear.

Despite these difficulties, *B. thuringiensis* has been the subject of intensive investigation using the tools of biotechnology. Because of the highly selective toxic activity associated with *B. thuringiensis*, biotechnologists see a potential to develop safe, effective, and economically feasible insect control agents. Research into new ways to use *B. thuringiensis* and/or its toxic products has focused in three general areas.

One area of interest centers on the problem of narrow host range. Although the advantages of such a narrow host range are clear, limited toxicity also restricts the use of *B. thuringiensis* as an insecticide. Wabiko et al. (1986), for example, compared the DNA and derived protein sequences of the crystal toxin genes from two strains of *B. thuringiensis* having different insect specificities. Interestingly, there were several areas of strong protein homology and three areas in which striking differences were clustered. The authors speculated that these three areas might be important for genetic determination of the insect specificity of the toxin. At the present time much effort is being exerted to understand the basis for insect specificity of the *B. thuringiensis* toxin in the belief that it may be possible to alter or expand the host range.

An alternative approach is that the toxin gene could be placed in another bacterium that would live longer in the environment, multiply, and, thus, remain in or on the insect's food supply for a longer period of time. In a recent review, Andrews et al. (1987) described three possible examples of this approach. One approach, for example, was to stabilize the subsp. *israelensis* toxin by cloning its gene into *Bacillus sphaericus*, an organism which is much more stable in water environments than *B. thuringiensis* var. *israelensis*. Another approach is to clone the toxin genes into pseudomonads, organisms known to adhere to certain parts of plants. Both living and killed strains containing toxin genes have been described. Also, it is possible to clone the subsp. *israelensis* toxin gene into cyanobacteria (Angsuthanasombat and Panyim, 1989). Such bacteria should both replicate in the water envi-

ronment and serve to make the mosquito larval food supply toxic.

Finally, the *B. thuringiensis* toxin gene can be directly cloned into and expressed in plant systems. Indeed, insect-resistant transgenic plants containing the *B. thuringiensis* toxin gene have been reported (Fischhoff et al., 1987).

Bacillus sphaericus

Although aerobic bacilli that produce round spores were described early in this century, the isolation of a strain that was pathogenic to mosquitoes and matched the description of *Bacillus sphaericus* did not occur until 1964 (Kellen and Meyer, 1964). This strain and subsequent isolates all possess the phenotypic characteristics typical of the species (see Chapter 76). Both pathogenic and nonpathogenic strains are aerobic bacilli producing round, terminally located spores that swell the sporangium. The spores vary in size and somewhat in shape (round to slightly oval) depending upon growth conditions. These bacteria are easily distinguished microscopically and physiologically from the other well-known mosquito pathogen, *B. thuringiensis* subsp. *israelensis*. The cells and spores of *B. sphaericus* are smaller than those of *B. thuringiensis* and the former species is much less active in its metabolism of a variety of substrates than is the latter. *B. sphaericus* is similar to *B. thuringiensis* in that the insect pathogenicity of each is mediated by the production of toxin.

Habitats and Isolation

The first isolation of a *B. sphaericus* mosquito pathogen was made from moribund, fourth-instar larvae of *Culiseta incidens* collected in California (Kellen and Meyer, 1964; Kellen et al., 1965). Additional isolates have also been obtained from dead mosquito larvae (Singer, 1973). More recently, pathogenic isolates have been obtained from black flies (Weiser, 1984), from caterpillars and grasshoppers (Lysenko et al., 1985), and from snails (de Barjac et al., 1988). These isolates were shown to be pathogenic for mosquitoes but lacked pathogenicity for the insects from which they were obtained. There was no indication that the snail isolate had been tested for pathogenicity to snails. *B. sphaericus* mosquito pathogens have also been obtained from mud taken from pools, from the soil of a dried stream bed (Brownbridge and Margalit, 1987), and from a loamy soil (de Barjac et al., 1988). Since this bacterium is not an obligate parasite and since there is no indication of marked differences in growth rates or metabolism between pathogenic and nonpathogenic

strains, it seems likely that pathogens may be recovered from the same soil and aquatic habitats that are occupied by the nonpathogens. However, it is known that the pathogenic strains can multiply in the cadavers of mosquito larvae that have been killed by the larvicidal toxin (Charles and Nicolas, 1986; Davidson et al., 1975, 1984). Thus, it is likely that larger populations of the pathogens may be found in aquatic habitats that contain dead or dying larvae.

Isolation of pathogenic *B. sphaericus* from dead larvae can be performed by crushing larvae in sterile water or buffer and streaking the homogenate onto a proteinaceous medium such as brain heart infusion agar or nutrient agar supplemented with 0.05% yeast extract. A medium containing amino acids or protein hydrolysate is required because *B. sphaericus* cannot utilize sugars as a carbon source (Russell et al., 1989). The inclusion of yeast extract in media is useful because many strains of *B. sphaericus* are auxotrophic for vitamins, frequently biotin and thiamine (White and Lotay, 1980). If isolations are made from mud or soil where the population of the pathogens might be lower than in larval cadavers, it is useful to heat the suspension to kill contaminating vegetative cells before streaking. *B. sphaericus* has been reported to be resistant to streptomycin, chloramphenicol, lincomycin, and bacitracin (Burke and McDonald, 1983; Kalfon et al., 1986; Yousten et al., 1985) and these antibiotics may be useful additions to selective media. A defined medium (BATS) utilizing arginine as the sole carbon and nitrogen source and streptomycin as a selective agent allowed growth of pathogenic strains of *B. sphaericus* but did not allow growth of 68% of the nonpathogenic strains tested. It also did not allow growth of several other aerobic, spore-forming bacteria.

BATS Defined Medium for Growth of *Bacillus sphaericus* Mosquito Pathogens (Yousten et al., 1985)

The medium contains per liter:

Na_2HPO_4	5.57 g
KH_2PO_4	2.4 g
$MgSO_4 \cdot 7H_2O$	0.05 g
$MnCl_2 \cdot 4H_2O$	0.004 g
$FeSO_4 \cdot 7H_2O$	0.028 g
$CaCl_2 \cdot 2H_2O$	0.002 g
L-Arginine	5.0 g
Thiamine	0.02 g
Biotin	2.0 µg
Streptomycin sulfate	0.1 g

The arginine, biotin, thiamine and streptomycin are prepared as a filter-sterilized stock solution. The Mg^{2+}, Mn^{2+}, Fe^{2+}, and Ca^{2+} salts are prepared as an acidified (0.03% [vol/vol] concentration of H_2SO_4), autoclaved stock solution. These two stock solutions are added to the autoclaved phosphate salts solution when the latter has cooled to 50°C.

At present there is no way to distinguish pathogenic strains from nonpathogenic strains of *B. sphaericus* by colony morphology or by any simple biochemical test. New isolates grown on solid media are examined microscopically for round spores and then grown in a liquid medium allowing good sporulation. This is followed by bioassay of the cell mass using susceptible mosquito larvae, e.g., *Culex quinquefasciatus*. Two rather similar media that have been widely used for obtaining good sporulation and concurrent toxic production are NYSM broth (Myers and Yousten, 1980) and MBS broth (Kalfon et al., 1983).

NYSM Broth (Myers and Yousten, 1980)

Nutrient broth	8.0 g
Yeast extract	0.5 g
$MgCl_2 \cdot 6H_2O$	0.2 g
$MnCl_2 \cdot 4H_2O$	10.0 mg
$CaCl_2 \cdot 2H_2O$	0.1 g

Identification

THE PLACE OF THE PATHOGENIC STRAINS WITHIN THE SPECIES.   Despite the phenotypic similarities and the same GC content (35–37 mol%) found in the pathogenic and nonpathogenic strains of *B. sphaericus*, large amount of genetic diversity appear to exist within this "species." The first indication of this diversity was given by Seki et al. (1978) and was greatly expanded to cover both pathogens and nonpathogens by Krych et al. (1980). Analysis using DNA hybridization revealed five major homology groups within the species. One of these groups (II) was subdivided into groups IIA and IIB. The former was composed of the seven mosquito pathogens included in the study, whereas all of the strains in the other homology groups were nonpathogenic. F. G. Priest (personal communication) used DNA hybridization to study additional pathogenic strains and obtained similar results. However, other than pathogenicity itself, no single phenotypic test allowed unambiguous differentiation of pathogens from nonpathogens. de Barjac et al. (1980) carried out numerical analysis of growth on 160 substrates to identify three groups; one of these contained nine pathogens and one nonpathogen and the other two groups contained 25 nonpathogens. Williston and Singer (1987) were able to separate six pathogens from the type strain of the species (a nonpathogen) and one other nonpathogen by electrophoretic analysis of aminopeptidase activity on four L-amino acid-beta naphthylamide substrates. F. G. Priest (personal communication) used a group of 13 phenotypic tests to successfully differentiate pathogenic strains from the closely related nonpathogenic strains of homology group IIB. He

found that adenine utilization was the single test that most often correlated with pathogenicity. Although the DNA homology studies clearly indicate that the mosquito pathogens are not the same species as the type strain of *B. sphaericus* (ATCC 14577), it is not clear if the pathogens are a separate species from the nonpathogens of homology group IIB. It is not yet known if the gene for the toxin responsible for pathogenicity is located on the chromosome or on a plasmid. If it is located on a plasmid, speciation of the pathogens would leave one in the same unsatisfactory position as is presently the case with *B. thuringiensis* and *B. cereus*.

DIVERSITY AMONG THE MOSQUITO PATHOGENIC STRAINS. The pathogenic strains of *Bacillus sphaericus* have been subdivided by serotyping (H antigens), bacteriophage typing, serotyping based upon antigenicity of the cell surface protein (the S layer), and the level of toxicity to mosquito larvae. These methods have shown remarkable agreement in the establishment of groups of strains.

Flagellar serotyping (H antigens) has identified six groups among the pathogenic strains (de Barjac et al., 1980; 1985, 1988). Three of these serotypes (H5a,5b; H25; and H6) contain highly toxic strains, whereas the other three serotypes contain weakly toxic strains. Serotype H6 is unique in that it also contains some nontoxic strains (de Barjac et al., 1988).

Several lytic bacteriophages have been isolated for the *B. sphaericus* pathogens. These phages have been used to group the strains, and the groups identified by phage typing correspond closely to the groups identified by flagellar serotyping (Yousten, 1984; Yousten et al., 1980). Phage group 3 contains the highly toxic strains of serotypes H5a,5b and H6.

The bacteriophages have been shown to attach to the bacterial cells via a high molecular weight glycoprotein (Lewis and Yousten, 1988). This protein is present in a fine, linear array (the S layer) on the cell surface of the mosquito pathogens (Lewis and Yousten, 1987). This is in contrast to the tetragonal arrangement of the S layer on the type strain, ATCC 14577. Immunological analysis of this protein revealed that it was distinct for each of the bacteriophage groups, although in several cases bacteriophages were neutralized by the protein from strains on which the phages failed to replicate. Thus, although the proteins are distinct for each phage group, they alone do not determine those groups. The gene encoding the S-layer protein from *B. sphaericus* 2362 has been cloned and sequenced (Bowditch et al., 1989). The nucleotide sequence indicated an open reading frame coding for a protein of 1,176 amino acids with a molecular weight of 125 kDa. A 30 amino acid leader peptide is cleaved from the 125-kDa protein during secretion resulting in a 122-kDa protein present on the cell surface. The 12 N-terminal amino acids of the 122-kDa S-layer protein and those of a 110-kDa larval toxin are identical. This larval toxin, which at one time was thought to be present in the parasporal body, thus appears to be derived from the S layer. Immunological studies also support this interpretation (Bowditch et al., 1989). Amino acid sequence comparisons of the 125-kDa protein with the S-layer proteins of other bacteria, revealed no sequence similarity to those of *Halobacterium halobium* or *Deinococcus radiodurans* but did show significant sequence similarity to the N-terminal portion of the "outer wall protein" of *Bacillus brevis* 47.

Ecophysiology

The major aspect of ecophysiology discussed below relates to the toxins produced by *B. sphaericus* strains that are mosquito pathogens. As indicated above, one of the mosquito larval toxins appears to be derived from the cell wall-associated S-layer glycoprotein. However, the toxin thought to be most important for pathogenicity is primarily located in a parasporal body or crystal (Davidson and Meyers, 1981). This inclusion body appears in the cell during the time of engulfment of the forespore by the forespore septum. Thin sections of the inclusions revealed a crystalline lattice structure having striations at a 6.3-nm interval (de Barjac and Charles, 1983; Yousten and Davidson, 1982). Unlike the paraspores of *B. thuringiensis*, which form outside the exosporium, those of *B. sphaericus* are partially enclosed by an elongated exosporium. This results in many of the paraspores remaining attached to the endospore upon lysis of the mother cell at the completion of sporulation. When paraspores were fed to mosquito larvae, the protein matrix of the paraspores rapidly dissolved in the alkaline midgut leaving a meshlike envelope in the shape of the paraspore (Yousten and Davidson, 1982). The composition of the parasporal envelope is unknown.

The matrix of the parasporal body is composed of two proteins: a 41.9-kDa protein that is toxic to mosquito larvae and to cultured mosquito cells, and a 51.4-kDa protein that is toxic to neither. Genes encoding these proteins have been cloned and sequenced from three highly toxic strains (Baumann et al., 1988; Berry and Hindley, 1987; Hindley and Berry, 1987, 1988). No sequence similarity was found between the *B. sphaericus* toxin and the Diptera- and Lepidoptera-active toxins of *B. thuringiensis* subsp. *israelensis* and *kurstaki,* respectively (Baumann

et al., 1988). The N-terminus of the toxin protein present in the paraspore is missing four amino acids as compared to the amino acid sequence deduced from the nucleotide sequence. It has been suggested that this deletion is produced by a *B. sphaericus* protease. Following ingestion of the 41.9-kDa toxin by mosquito larvae, the larval gut proteases remove an additional six amino acids from the N-terminus and approximately 20 amino acids from the C-terminus. This proteolysis enhanced the cytotoxicity of the protein for cultured mosquito cells (Broadwell and Baumann, 1987; Davidson, 1986; Davidson et al., 1987a). Interestingly, when the cloned 41.9-kDa toxin was produced in *E. coli*, it was not toxic to larvae unless it was combined with the 51.4-kDa (nontoxic) protein (Baumann et al., 1988).

In addition to the highly toxic strains, which produce a parasporal body containing the 41.9-kDa toxin, there are low-toxicity strains that do not produce a paraspore (Davidson and Myers, 1981). Probes that utilize the nucelotide sequence of the 41.9-kDa toxin did not detect these sequences in dot blots of the low toxicity strains (D. Wilcox, personal communication). The nature of the toxicity in these low toxicity strains is unknown and the possible relationship to the 110-kDa S layer-derived toxin remains to be determined.

The *B. sphaericus* toxin affects a variety of mosquito species from several genera but, unlike the toxin(s) from *B. thuringiensis* subsp. *israelensis,* it is not toxic to black fly larvae. It is much more toxic for mosquito larvae of the genus *Culex* than it is for *Aedes aegypti* (though certain other species of *Aedes* are quite sensitive). The degree of sensitivity to the toxin may be related to the ability of the toxin to bind to midgut cells in the larvae. Good binding of fluorescein-labeled toxin was observed in the gut of *Culex pipiens,* but there was no binding in the gut of *Aedes aegypti* (Davidson, 1989). The effects of the toxin have been studied both in mosquito larvae (Charles, 1987; Davidson, 1979; Singh and Gill, 1988) and in cultured mosquito cells (Davidson, 1986; Davidson and Titus, 1987; Davidson et al., 1987b). Although the pathological effects have been reported in detail for events occurring at the organ and cellular level, the mechanism of action of the toxin at the subcellular or molecular level is unknown.

Genetics

Although lytic bacteriophages have been isolated that use the mosquito pathogens as hosts, temperate phages capable of carrying out transduction have not been described. The phenomenon of lysogeny has not been demonstrated in these bacteria.

The procedures used to induce competence in *Bacillus subtilis* have not proven successful for carrying out transformation in *B. sphaericus*. However, plasmid-mediated transformation of polyethylene glycol (PEG)-treated protoplasts was performed by McDonald and Burke (1984). An improved PEG-protoplast transformation procedure using restriction-deficient strains of *B. sphaericus* 1593 produced about 1×10^3 transformants/μg of pUB110, pLT105, or pAMB1 DNA (Taylor and Burke, 1989). W. Burke (personal communication) also used electroporation for the introduction of plasmid DNA into *B. sphaericus*. Restriction and modification systems were suggested by the fact that in *B. sphaericus* 1593 the transformation efficiency was 10^4-fold greater when the plasmid DNA, pUB110, was isolated from *B. sphaericus* rather than *B. subtilis*. This was confirmed by agarose gel electrophoresis of pUB110 and PBC16 incubated with lysates from *B. sphaericus* and *B. subtilis*. The results indicated the restriction system is an isoschizomer of *Hae* III (McDonald and Burke, 1984; Burke, personal communication). Cloning vectors for the mosquito pathogenic strains are being constructed (Norton et al., 1985).

Conjugation between *B. sphaericus* 1593 and *Enterococcus (Streptococcus) faecalis* using the method of filter mating was carried out by Orzech and Burke (1984). The transfer process was more effective between *E. faecalis* and *B. sphaericus* than between strains of *B. sphaericus*. It was unaffected by either endogenous restriction enzyme activity or by the presence of exogenous DNase.

The reports of naturally occurring plasmids in the toxin-producing strains of *B. sphaericus* have provided somewhat conflicting information. Davidson et al. (1982) reported the presence of a single large plasmid in the highly toxic strains 1593 and 1881 but no plasmids in strains 1691 or 2362. Abe et al. (1983) found a single large plasmid in strain 1881 but no plasmids in strains 1593 or 1691. In the weakly pathogenic strain Kellen K, Davidson et al. (1982) found no plasmids but Abe et al. (1983) found five. Singer (1987) reported an approximately 75-MDa plasmid in the highly toxic strains 1593, 2362, and 2297 as well in the less toxic strain SSII-1. Strain 2297 also contained two smaller plasmids (3.4 and 3.2 MDa). A. A. Yousten (unpublished observations) obtained results similar to those of Singer. The approximately 75-MDa plasmid seems to be common among the highly toxic strains, but the plasmid is not restricted to them because it is also present in the much less toxic strain SSII-1. In *B. thuringiensis* the gene(s) for the toxin(s) are usually present on plasmids. However, this determination has not yet been made for the mosquito larval toxin of *B. sphaericus*.

Biotechnological Applications

The major biotechnological application of mosquito-pathogenic strains of *B. sphaericus* has been as a biological control agent.

Following ingestion of a mixture of spores and toxin-containing paraspores, the number of viable spores in the gut decreases until the death of the larva, which usually occurs about 24 h after ingestion of the toxin. At that time some unknown fraction of the spores germinate, grow vegetatively, and sporulate in the larval cadaver. This results in about a 10–100-fold increase in the number of spores compared to the number originally consumed (Charles and Nicolas, 1986; Davidson et al., 1984). Each larval cadaver seems capable of producing about 10^5–10^6 spores. This recycling of the spores in the cadaver may be related to the prolonged larval control reported for this bacterium compared to *B. thuringiensis* subsp. *israelensis* when sporulated cultures of these bacteria have been sprayed into water to control mosquito breeding (Singer, 1987). The attractiveness of *B. sphaericus* as a mosquito larvicide is related to its extended persistence in the water and to the unrelatedness of its toxin to that of *B. thuringiensis* subsp. *israelensis*. This latter characteristic might make it a useful alternative should resistance to the *B. thuringiensis* toxin appear. Efforts are being made to introduce toxin genes from *B. thuringiensis* subsp. *israelensis* into *B. sphaericus* with the goal of extending the lethal effects of the latter bacterium to *A. aegypti,* a species of mosquito quite resistant to the usual *B. sphaericus* toxin. This would also extend the environmental persistence of the *B. thuringiensis* toxin. An additional attempt to extend environmental persistence has been made by de Marsac et al. (1987), who cloned an 8.6-kb fragment of *B. sphaericus* 1593M DNA into the cyanobacterium *Anacystis nidulans R2,* using the shuttle vector pUC303. The resulting transformant was toxic to mosquito larvae, though at least 1000-fold less toxic than the *B. sphaericus* parent.

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CHAPTER 1.2.18

The Genus *Bacillus*—Medical

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Of the 34 species of the genus *Bacillus*, the two of greatest medical importance are *B. anthracis*, the causative agent of anthrax, and *B. cereus*, which causes food poisoning. Nonanthrax *Bacillus* species, including *B. cereus*, also cause a wide variety of other infections, and they are being recognized with increasing frequency as significant pathogens in humans. Species which have caused human disease include *B. cereus*, *B. alvei*, *B. megaterium*, *B. coagulans*, *B. laterosporus*, *B. subtilis*, *B. sphaericus*, *B. circulans*, *B. brevis*, *B. licheniformis*, *B. macerans*, *B. pumilus*, and *B. thuringiensis*. After *B. anthracis*, *B. cereus* is the most frequent human pathogen. *B. subtilis* has been used as a synonym for aerobic sporeformers other than *B. anthracis*, and many isolates described as *B. subtilis* in the early literature were probably *B. cereus* (Weinstein and Colburn, 1950).

Anthrax

Anthrax has a long and fascinating history. The disease in cattle is described in Egyptian and Mesopotamian writings of around 5000 B.C. (Dürst et al., 1986) and it may have been responsible for two of the plagues visited upon the Egyptians and their cattle in 1491 B.C. (the "murrain of beasts" and the "plague of boils and blains"), described in the ninth chapter of Exodus. In the *Georgics,* written around 37 B.C. Publius Vergilius Maro (Virgil) refers to anthrax infection in sheep, cattle, horses, and oxen, and in men handling wool from infected sheep (Dirckx, 1981). As a cause of "black bane" and "murrain," it caused heavy losses of cattle and sheep in England and Europe in Saxon and Medieval times, and may have contributed to the Black Death that ravaged Europe in the mid 14th century (Twigg, 1984). In 1613, there were approximately 60,000 human deaths from anthrax in southern Europe.

In a series of papers written in 1863 to 1864, Casimir Davaine presented evidence linking anthrax in animals and humans with certain bacteria found in the blood and tissues (Davaine, 1863a, 1863b, 1864; Davaine and Raimbert, 1864). In 1876, Robert Koch visualized anthrax bacilli in the blood and tissues of animals dying of anthrax, demonstrated that the bacillus could form spores that remained viable for long periods in hostile environments, and claimed credit for proving that the bacillus is the causative agent of anthrax (Koch, 1877). Louis Pasteur, in a series of papers beginning in 1877, isolated the anthrax bacillus in pure culture from animals, propagated the organism in serial cultures, and reproduced the disease in experimental animals (reprinted in Vallery-Radot, 1939). It was thus Pasteur who fulfilled "Koch's postulates" in establishing a causal relationship between the anthrax bacillus and anthrax (Carter, 1988).

Because of vaccination programs and improved practices of raising livestock, anthrax has become a rare disease in many industrialized countries; an average of only two or three cases per year have been reported in the United States during the past 20 years. Most cases in industrialized countries are associated with exposure to animal products, especially goat hair, imported from Iran, Turkey, Pakistan, and Sudan, where anthrax remains common among domestic livestock. Worldwide anthrax remains an important disease, with an estimated 20,000 to 100,000 human cases occurring each year (Glassman, 1958). Recent outbreaks of human infection have been reported from Zimbabwe (Davies, 1982, 1983, 1985; Turner, 1980); Turkey (Doganay et al., 1986); Thailand (Sirisanthana et al., 1984); Bangladesh (Samad and Hoque, 1986); Afghanistan (Arya et al., 1982); Tanzania (Webber, 1985); Ethiopia (Seboxa and Goldhagen, 1988); and Uganda (Ndyabahinduka et al., 1984). During the civil war in Zimbabwe in 1979 to 1980, when medical services and animal vaccination programs were disrupted, more than 6,000 human cases occurred, with approximately 100 deaths.

Because of the extraordinary stability of the spores of *B. anthracis* and their virulence following inhalation or ingestion, anthrax has received much consideration as a potential agent for use

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This chapter was taken unchanged from the second edition.

in biological warfare. In 1979, a large outbreak of anthrax, possibly involving more than 1,000 people, occurred in and around Sverdlovsk, a large industrial city in the Ural Mountains of the Soviet Union (Wade, 1980). It was suspected that this outbreak might have resulted from an accident of some kind, possibly an explosion at a secret military laboratory where a biological warfare agent was being prepared. This assertion was denied by the Soviet Union, and 10 years later a delegation of Soviet officials visited the National Academy of Sciences in Washington, D.C. to explain that the epidemic had consisted primarily of cases of intestinal anthrax, traced to a large lot of contaminated bone meal used as cattle feed. According to Soviet officials, the human victims became ill after eating infected meat purchased on the black market (Marshall, 1988).

Habitat and Ecology

The spores of *B. anthracis* are extremely resistant to heat, cold, desiccation, and chemical disinfection and may remain viable in the soil of pastures for many decades. Certain fields in Europe have been associated with repeated epizootics of anthrax in grazing animals for more than 100 years. Although the soil is the ultimate reservoir of *B. anthracis*, its ecology is complex (Van Ness, 1971). A vegetative phase may be required in which the organisms multiply to a density sufficient to infect grazing animals. In suitable limestone soils, with pH above 6.0 and ambient temperature above 15.5°C, a spore-vegetative cell-spore cycle may be maintained indefinitely and livestock grazing on such pastures may become infected. In unsuitable areas, perhaps because of acidity or intense bacterial competition, both organisms and spores may be eliminated rapidly. "Incubator areas" may develop in depressions where water has stood long enough to devitalize or kill grass. Flooding of these areas may result in dispersal of spores over a wide expanse. Cattle dying with anthrax commonly discharge large numbers of anthrax bacilli from the nose, mouth, and intestinal tract, thus returning organisms to the soil. Sporulation and dispersal of spores may also occur when the carcass of an animal dying of anthrax is opened in the field, either for necropsy or for butchering in countries where such animals provide meat for human consumption (McKendrick, 1980). Pasteur et al. 1880 showed that earthworms can return anthrax spores from the buried carcasses of animals dying of anthrax to the surface of the soil. Mechanical dispersal of spores over short distances may occur via flies, and over long distances by vultures which have fed on the carcasses of infected animals (McKendrick, 1980; Davies, 1983).

Acquisition of anthrax by animals and humans occurs via spores; vegetative forms probably play no significant role in its transmission. Grazing animals often acquire anthrax during periods of drought, when the herds are forced to graze on spiny vegetation close to the ground and abrasions on the lips allow deposition of spores in the tissues; ingestion of spores along with vegetation is another route of infection. Fertilizers made from contaminated animal bones, hay, water, and animal feeds are additional sources of infection in animals. Biting flies may transmit anthrax among animals in some tropical countries but probably do not constitute an important source of infection worldwide.

Human cases of anthrax are acquired via contact with infected animals or animal products and may be classified as *agricultural* or *industrial*. Agricultural cases predominate in developing countries where there is an extensive agricultural economy and anthrax is enzootic in livestock. Outbreaks of human anthrax are almost always preceded by epizootics in cattle, as occurred in the large epidemic of human anthrax in Zimbabwe in 1979 to 1980. Nearly all cases of agricultural anthrax are of the cutaneous form and result from handling infected carcasses or animal products. In countries where animals dying of anthrax are consumed by humans, occasional cases of intestinal and oropharyngeal anthrax occur. An outbreak of 24 cases of oropharyngeal anthrax, and many cases of the cutaneous form, occurred in northern Thailand in 1982 as a result of individuals eating the meat of sick cattle and water buffaloes imported on the hoof from Burma (Sirisanthana et al., 1984). The role of biting insects in transmission of anthrax to humans remains controversial. Stable flies (*Stomoxys* spp.) and two species of *Aedes* mosquitoes have been shown to be capable of transmitting anthrax from infected guinea pigs to uninfected guinea pigs and mice (Turell and Knudson, 1987). During the 1979 to 1980 epidemic in Zimbabwe, several lines of evidence suggested that transmission occurred via the bites of stable flies and horse flies (Tabanidae): 1) the epidemic coincided with an explosive increase in the population of horse flies; 2) in children, who had little exposure to infected animal carcasses, there was an increased incidence of lesions on the head, neck, and face, where insect bites were common; and 3) a number of patients remembered an insect bite at the site where an anthrax lesion developed later (McKendrick, 1980; Davies, 1983). Person-to-person spread occurs very rarely if at all; there were no cases among the nursing staff and no evidence of cross-infection among patients during the Zimbabwe epidemic.

Industrial anthrax results from contact with spore-contaminated materials such as goat hair,

animal hides, wool and bones used in manufacturing processes, or finished products such as shaving brushes, bongo drums, and saddle blankets. Most cases are of the cutaneous type, but occasional cases of inhalation anthrax (woolsorter's disease) are seen (Brachman, 1980). Nearly all such cases occur in workers exposed to aerosols of anthrax spores during early processing of imported goat hair or hides, or less commonly wool. Very rarely the disease occurs in laboratory workers or in artisans working with imported animal hair or skins. There have been only two cases of inhalation anthrax in the United States in the last 20 years (18 cases during this century).

Anthrax in Animals

Anthrax is primarily a disease of herbivorous animals, especially cattle, sheep, horses, goats, and wild herbivores, although nearly all mammals are susceptible to the disease to some degree. Omnivores such as man and swine, and carnivores such as dogs, possess greater natural resistance to anthrax than do herbivores (Knudson, 1986).

Three forms of anthrax, peracute, acute/subacute, and chronic, are recognized in animals (Hunter et al., 1989). The peracute form is seen mainly in ruminants, especially cattle, sheep, and goats. The animals may develop cerebral anoxia and pulmonary edema, and death may occur suddenly.

Acute and subacute anthrax are characterized by fever, depression, convulsions, dyspnea, and hemorrhage from the mouth, nose, and anus, with death occurring approximately 24 h after the initial signs of illness. This form of anthrax is seen primarily in cattle, sheep, and horses. Pathologic findings include hemorrhage and edema in many different organs. In cattle and sheep, the spleen is enlarged with a "blackberry jam" appearance.

In chronic anthrax, seen most often in swine but also in horses, dogs, and cattle, the typical clinical signs are edema of the tongue and pharyngeal tissues with dyspnea and serosanguinous discharge from the mouth. Death due to asphyxia may occur. In swine, an intestinal form of chronic anthrax has been reported. At necropsy, edema and hemorrhage of the involved tissues is found.

Anthrax in Humans

Cutaneous anthrax accounts for approximately 95% of cases. Spores of *B. anthracis* are deposited underneath the epidermis via a minor cut or abrasion or insect bite. These spores then germinate, and the vegetative cells produce toxin. Following an incubation period of 2 to 7 days, a small papule which is often pruritic appears at the site where spores have been inoculated. This enlarges and within 24 to 48 h develops into an ulcer surrounded by vesicles. Eventually the characteristic black eschar develops, surrounded by edema which is often striking, especially with lesions on the face and neck. (The name "anthrax" is derived from the Greek *anthrakos*, which means "coal," as does *charbon*, the French term for the disease.) The lesion is painless. Most patients are afebrile and the white blood cell count is usually normal or only slightly elevated. More than 90% of lesions occur on exposed areas such as the face, neck, hands, and arms. Lymphangitis and regional lymphadenopathy may be present.

Cutaneous anthrax is usually a self-limiting disease and lesions heal spontaneously in 80 to 90% of cases (Knudson, 1986). In untreated cases, death may occur in 10 to 20% due to airway obstruction (especially with lesions of the face or neck) (Davies, 1982), bacteremia with shock and renal failure, or meningitis. Early and appropriate antibiotic therapy reduces the case fatality rate to less than 1%.

Internal forms of anthrax infection are much more difficult to diagnose, and the case fatality rate is much higher than in the cutaneous form, even with prompt and appropriate antibiotic therapy. *Inhalation* anthrax accounts for up to 5% of cases (Brachman, 1980). Most cases result from the inhalation of spores of *B. anthracis* during the processing of contaminated goat hair or wool. The infectious dose is high, probably on the order of 50,000 spores. After inhalation, the spores are ingested by alveolar macrophages and carried to the hilar lymph nodes. Germination and multiplication of the vegetative forms, with production of toxin, occur in the hilar nodes, resulting in edema and hemorrhage in the nodes and surrounding mediastinal structures. Eventually the bacilli may gain access to the blood via the lymphatics, with sepsis and subsequent metastatic spread. The clinical picture is typically that of a biphasic disease. The initial stage, which lasts 2 or 3 days, has an insidious onset with mild fever, malaise, fatigue, myalgia, nonproductive cough, and sometimes a sensation of precordial pressure, and is often mistaken for a viral respiratory infection. The second stage begins suddenly with acute dyspnea, cyanosis, diaphoresis, and stridor with rapid progression to a moribund state and death within 24 h. The characteristic roentgenographic picture is one of mediastinal widening due to lymph node enlargement. At autopsy hemorrhagic mediastinitis and lymphadenitis are found, often with little involvement of the lung parenchyma. Of the 18 cases of inhalation anthrax reported in the American literature since 1900, 16 were fatal.

Intestinal anthrax is very rare in humans and has never been documented in the United States (Nalin et al., 1977; Jena, 1980; Ndyabahinduka et al., 1984; Bhat et al., 1985). The disease follows ingestion of heavily contaminated, undercooked meat. Multiplication of organisms and production of toxin in the bowel wall and in mesenteric lymph nodes leads to edematous and hemorrhagic enteritis and lymphadenitis. The clinical picture is variable. Some cases present with gaseous distention and multiple fluid levels suggesting small bowel obstruction, with development of peritonitis. At surgery or autopsy, severe edema and gangrene of the bowel with multiple perforations and black necrotic areas may be found. The mesentery may be grossly edematous and hemorrhagic mesenteric lymphadenitis may be present. Other patients present with rapid development of hypovolemic shock and massive hemorrhagic ascites. The case fatality rate is 25 to 50% regardless of treatment.

Anthrax meningitis, another rare form of the disease, may occur as a result of the bacteremia which can accompany cutaneous or inhalation anthrax (Koshi et al., 1981; Trivedy, 1981; Chandramukhi et al., 1983; Dürst et al., 1986). In approximately one-half the cases, meningitis results from hematogenous dissemination from a cutaneous lesion. Nearly one-half of patients with inhalation anthrax develop meningitis. The clinical picture is that of a rapidly progressive acute bacterial meningitis. The case fatality rate, even with antibiotic treatment, is nearly 100%. At autopsy an acute hemorrhagic leptomeningitis, sometimes with subarachnoid, cortical or deep hemorrhages, or diffuse encephalitis, is found.

Oropharyngeal anthrax has occurred following ingestion of infected beef or water buffalo meat (Sirisanthana et al., 1984; Doganay et al., 1986). The primary lesion on the tongue, tonsil, or pharyngeal wall exhibits central necrosis and ulceration with surrounding edema and congestion. The neck on the involved side is markedly swollen with enlargement of cervical lymph nodes. The case fatality rate in patients treated with penicillin has varied from 15 to 50%.

A few cases of anthrax have been reported in cases of attempted abortion with spore-infected date stems or twigs, or following delivery in a stable, leading to hemorrhagic metritis, parametritis, and oophoritis, most ending in septicemia and death (Dutz and Kohout-Dutz, 1981).

Treatment and Prevention

The prognosis in cutaneous anthrax is good; 80 to 90% of cases resolve without specific antibiotic therapy. However, because of the potential complications of malignant edema, septicemia, shock, renal failure, and meningitis, patients with anthrax should receive prompt treatment, on the basis of clinical suspicion or visualization of organisms in Gram stains of specimens taken from the lesions. Treatment should not be delayed until bacteriologic confirmation is obtained. Penicillin G is the drug of choice in the treatment of anthrax (Knudson, 1986). An appropriate dosage regimen for treatment of cutaneous anthrax is two million units intravenously every 6 h for 2 to 4 days, until substantial improvement has occurred. At this time therapy can be changed to oral penicillin V, and treatment continued for a total of 7 to 10 days. Cultures of vesicle fluid become negative within a few hours after initiation of therapy with penicillin, but the lesion continues to evolve to the eschar phase. Systemic manifestations and local edema usually resolve promptly. Appropriate alternative choices for patients allergic to penicillin are erythromycin, tetracycline, and trimethoprim/sulfamethoxazole. Corticosteroids are recommended by many investigators when massive edema is present, and tracheostomy should be performed in patients with airway obstruction due to massive edema. Local surgical manipulation is ineffective and is contraindicated.

In patients with inhalation or gastrointestinal anthrax, severe sepsis or anthrax meningitis, the prognosis is poor regardless of treatment. In guinea pigs with experimental infection, once the level of bacteremia reaches three million organisms per milliliter of blood, eradication of the infection with antibiotics does not prevent a fatal outcome due to toxemia. This observation probably holds true for humans with septicemic anthrax (Keppie et al., 1955). The best hope is to begin therapy with high doses of penicillin G (12–20 million units per day, given intravenously in divided doses every 4 h) as soon as this type of infection is suspected. There is some experimental evidence that the combination of penicillin plus streptomycin (1–2 g per day intramuscularly) may be more effective in septicemic anthrax than penicillin alone (Lincoln et al., 1964). Chloramphenicol is not effective in the treatment of experimental septicemic anthrax and should not be relied upon for the treatment of systemic forms of infection.

In most countries, livestock at risk are immunized annually with commercial vaccines consisting of viable spores of the Sterne strain of *B. anthracis*, a nonencapsulated toxigenic variant which lacks the pX02 plasmid (Hambleton et al., 1984). This vaccine is effective and safe for use in many domestic animals (cattle, sheep, pigs, camels, buffaloes, and elephants) but progressive disease due to the vaccine strain has been observed in goats and llamas (Cartwright et al.,

1987; Sterne, 1988). The human anthrax vaccine licensed in the United States is produced by the Michigan Department of Public Health, and consists of aluminum hydroxide-adsorbed culture filtrate of the nonencapsulated toxigenic V770-NP1-R strain (composed primarily of protective antigen) (Ivins et al., 1986). Although guinea pigs immunized with the human vaccine produce high titers of antibody to protective antigen, only animals vaccinated with the Sterne strain are completely protected against challenge with highly virulent strains of *B. anthracis* (Little and Knudson, 1986). There appear to be at least two immunogenic vegetative cell-surface antigens present in the Sterne live vaccine which are not present in the human vaccine (Ezzell and Abshire, 1988). Modern studies have shown that the Pasteur vaccine strains (nontoxigenic, encapsulated) neither elicit antibody titers to any of the toxin components nor provide protection against challenge with virulent spores (Ivins et al., 1986). New approaches to development of more effective vaccines for human use include identification and incorporation of essential antigens and epitopes and transposon-induced mutagenesis to produce live vaccine candidate strains (Ivins and Welkos, 1988; Ivins et al., 1988).

When anthrax occurs in a herd, the source of infection must be determined and eliminated, and survivors should be treated prophylactically with antibiotics and the vaccination status brought up-to-date (Hunter et al., 1989). The affected farm should be quarantined for a period of at least 2 weeks following the last death from anthrax. Milk from unvaccinated febrile dairy cattle in the herd should be discarded and sick animals should be isolated. Animals dying of anthrax should be cremated or buried deeply after being covered with lime. Necropsy of dead animals should not be carried out in the field because of the risk of contaminating the soil with spores. Cases of anthrax in animals and humans should be reported to the appropriate health authorities.

Virulence Factors of *B. anthracis*

Fully virulent strains of *B. anthracis* possess two unique virulence factors: a poly-D-glutamic acid capsule (Green et al., 1985), which inhibits phagocytosis, and a tripartite toxin composed of protective antigen (PA), edema factor (EF) and lethal factor (LF) (Stephen, 1981; Leppla, 1988). Capsules are produced by virulent strains of *B. anthracis* growing in vivo, and by cells grown on media containing serum or bicarbonate or both and incubated in a CO_2-enriched atmosphere. The colonies of organisms that produce capsules appear mucoid, whereas colonies of organisms grown in the absence of serum or bicarbonate

fail to produce capsules and appear rough (Green et al., 1985).

The existence of an anthrax toxin was first clearly demonstrated by Smith et al. (1955), who produced local edema and death with injections of sterile plasma from infected guinea pigs. Studies by American and British investigators during the ensuing decade showed that the toxin contained three separate components (Stephen, 1981). The individual toxin components have no known biological effects when administered alone, but EF injected with PA into the skin of rabbits or guinea pigs causes local edema, and PA injected with LF into rats causes death in as little as 60 min. EF and LF are mutually inhibitory, suggesting that they compete for the same binding site on PA (Ezzell et al., 1984). Protective antigen, so-called because its injection into experimental animals results in protective immunity, binds to cell-surface receptors to produce an uptake system that can be used by both EF and LF to gain access to the cytoplasm. "Edema toxin" (EF + PA) and "lethal toxin" (LF + PA) thus resemble the A-B enzyme-binding structures characteristic of many well-studied bacterial toxins (Middlebrook and Dorland, 1984). After PA, analogous to the B chain, binds to a specific membrane receptor on the surface of a eukaryotic cell, it is cleaved at a single site, exposing a binding site for the other toxin component. The membrane-bound fragment of PA then binds to EF or LF and mediates the entry of the active moiety into the cytosol from an acidified endocytic vesicle. This fragment of PA has been shown to have channel-forming activity in planar phospholipid bilayers, and such channels may provide an aqueous pore in the endosomal membrane through which EF and LF enter the cell (Blaustein et al., 1989).

EF has been found to be a calmodulin-dependent adenylate cyclase that elevates cyclic AMP levels approximately 200-fold above normal in Chinese hamster ovary cells (Leppla, 1982, 1984). Local edema, a typical sign of anthrax, may be directly related to adenylate cyclase activity associated with EF. The increase in intracellular cAMP caused by this toxin may lead to edema in a manner analogous to the loss of water in the intestine caused by cholera toxin, which also increases intracellular cAMP (Leppla, 1982). EF + PA also inhibits phagocytosis of anthrax bacilli by polymorphonuclear leukocytes, and blocks both particulate and phorbol myristate acetate-induced chemiluminescence in polymorphonuclear leukocytes (O'Brien et al., 1985). These effects are accompanied by an increase in intracellular cAMP levels. The findings suggest that EF + PA may increase host susceptibility to anthrax by suppressing polymorphonuclear leukocyte function. The

dependence of EF activity on calmodulin, a substance found only in eukaryotic cells, suggests that EF did not evolve from a bacterial enzyme but from a eukaryotic adenylate cyclase, the gene for which was adventitiously transferred into *B. anthracis* and retained because it made the bacteria more virulent (Leppla, 1984). EF also exhibits substantial DNA homology and immunological relatedness with the *Bordetella pertussis* adenylate cyclase toxin (Escuyer et al., 1988). However, cytochalasin D, which interferes with endocytosis, and both ammonium chloride and chloroquine, which prevent acidification of endosomes, block intoxication of Chinese hamster ovary cells by EF, but not by pertussis adenylate cyclase toxin (Gordon et al., 1988).

The mechanism of action of LF is poorly understood. LF is lethal for many species of experimental animals and is assumed to be the major factor causing death in anthrax. No enzymatic activity has yet been associated with LF, and the nature of the intracellular target of LF is unknown. Certain macrophages and a mouse macrophage-like cell line are lysed at low concentrations of LF, but most cells are resistant (Singh et al., 1989). Resistant cells may either lack the intracellular target of LF or fail to process LF to an active form. There is evidence that PA may also be required for full activity of LF at a stage subsequent to endocytosis. LF is also calmodulin-dependent, and calcium is required at several steps for the expression of its effect (Bhatnagar et al., 1989).

Virulent strains of *B. anthracis* contain two large plasmids, pX01 and pX02 (Mikesell et al., 1983; Green et al., 1985; Kaspar and Robertson, 1987). Both plasmids are required for full pathogenicity, and strains which contain only one of these plasmids are avirulent. pX01 (174 kb pairs) encodes all three components of the anthrax toxin and pX02 (95 kb pairs) encodes the poly-D-glutamic acid capsule. The avirulent Sterne vaccine strain, which is pX01$^+$/pX02$^-$, produces toxin but no capsule and is used effectively as a live veterinary vaccine. The heat-attenuated Pasteur vaccine strains form capsules but are unable to produce toxin. Some rough variants of the Pasteur strains lack pX02, but others contain this plasmid. Reversion to mucoid colonies may be observed in strains that have retained pX02, but reversion of those cured of the plasmid is never observed. Pasteur probably cured his strains of plasmid pX01 by heat attenuation to produce his vaccine for immunization of cows and sheep.

The genes encoding all three toxin components have been cloned in *Escherichia coli* (Vodkin and Leppla, 1983; Robertson and Leppla, 1986; Mock et al., 1988; Tippetts and Robertson, 1988), and the base sequences for the PA and EF genes have been determined (Robertson et al.,

1988; Welkos et al., 1988). The PA gene has been cloned in *B. subtilis*, and immunization with the live recombinant strains protected guinea pigs from lethal challenge with virulent *B. anthracis* spores (Ivins and Welkos, 1986).

Bacillus cereus Food Poisoning

B. cereus has been recognized as an agent of food poisoning for the past several decades (Hauge, 1955). Between 1972 and 1986, 52 outbreaks of food-borne disease associated with *B. cereus* (1.9% of the total) were reported to the Centers for Disease Control (CDC). *B. cereus* strains cause two types of food-poisoning syndromes. These are true intoxications rather than infections. One type is characterized by nausea and vomiting (100%) and abdominal cramps (100%) and has an incubation period of 1 to 6 h (Terranova and Blake, 1978). This type has been referred to as the "emetic syndrome" or the short-incubation form. It resembles *Staphylococcus aureus* food poisoning in symptomatology and the short incubation period.

The second type of *B. cereus* food poisoning is manifested primarily by abdominal cramps (75%) and diarrhea (96%) with an incubation period of 8 to 16 h (Terranova and Blake, 1978). Diarrhea may be small volume or profuse and watery. This type is the "diarrheal syndrome" or long-incubation form (Mortimer and McCann, 1974) and resembles food poisoning caused by *Clostridium perfringens*. The illness usually lasts less than 24 h. In a few patients symptoms may last longer (2–10 days) (reference is not an exact matchGiannella and Brasile, 1979).

The short-incubation form is caused by a preformed heat-stable enterotoxin of molecular weight less than 5,000 which is produced by some *B. cereus* strains and which causes vomiting when fed to monkeys (Melling et al., 1976). The mechanism and site of action of this toxin are unknown (Turnbull et al., 1979b). The long-incubation form of illness is also enterotoxin-mediated. This toxin is produced in vivo (accounting for the longer incubation period), is heat-labile, and has a molecular weight of approximately 50,000 (Turnbull et al., 1979b). It activates intestinal adenylate cyclase and causes intestinal fluid secretion (Turnbull, 1976). It appears to have cytotoxic properties in rabbit small intestine and in guinea pig skin (Turnbull, 1976; Turnbull et al., 1979b).

B. cereus food poisoning occurs year round and is without any particular geographic distribution. The short-incubation form is most often associated with fried rice that has been cooked and then held at warm temperatures for several hours. The disease is often associated with

Chinese restaurants, especially in Europe, where leftover rice is often held overnight at room temperature because cooked rice which has been refrigerated is difficult to toss into beaten eggs. Growth of *B. cereus* in rice under similar experimental conditions has been documented (Gilbert et al., 1974). In one reported outbreak, the vehicle was macaroni and cheese made from powdered milk that was the source of the organism (Holmes et al., 1981). The short-incubation type is most often caused by serotype 1 strains (Gilbert and Parry, 1977) probably because they are more heat resistant than strains of other serotypes (Parry and Gilbert, 1980).

Long-incubation *B. cereus* food poisoning is frequently associated with meat or vegetable-containing foods. *B. cereus* contaminates raw meats, vegetables, and milk products. It has been isolated from 50% of dried beans and cereals (Blakey and Priest, 1980) and from 25% of dried foods such as spices, seasoning mixes, and dried potatoes (Kim and Goepfert, 1971). An outbreak of the long-incubation form was traced to a "meals-on-wheels" program in which food was held at and above room temperature for a prolonged period (Jephcott et al., 1977).

The short-incubation or emetic form of *B. cereus* food poisoning is diagnosed by the isolation of *B. cereus* from the incriminated food. The long-incubation or diarrheal form is diagnosed by isolation of the organism from stool and food. Isolation from stools is not sufficient unless negative stool cultures are obtained from a control group (Terranova and Blake, 1978). Fourteen percent of healthy adults have been reported to have transient gastrointestinal colonization with *B. cereus* (Ghosh, 1978). Serotyping may be of value in identifying a common source but is not readily available. Plasmid analysis recently proved useful in the epidemiological investigation of an outbreak of *B. cereus* gastroenteritis associated with the consumption of beef stew at a nursing home (DeBuono et al., 1988).

Because *B. cereus* gastroenteritis is generally a benign, self-limited illness, antimicrobial agents are of no value in management. Since bacteria grow best at temperatures ranging from 40 to 140°F, infection may be prevented if cold food is refrigerated and if hot food is held at greater than 140°F before serving.

Several methods have been described for production of the diarrheal toxin. Growth of *B. cereus* in brain heart infusion (BHI) broth with 0.1% glucose (pH = 7.4) is simple (Spira and Goepfert, 1972). High speed centrifugation is used to remove organisms and the supernatant is examined serologically by a modified microslide gel double-diffusion method for the identification of *B. cereus* diarrheal antigen (Crowle, 1958). Conditions for production of the emetic toxin are not as well defined. Because of the association of rice-containing foods with the emetic form of *B. cereus* food poisoning, most media for demonstrating emetic activity have contained rice (Melling et al., 1976). Autoclaved rice is soaked in saline. An overnight culture of rice is liquefied by diastase then dialyzed for 24 h at 4°C against 10% polyethylene glycol (Melling et al., 1976). The addition of chicken, beef, or egg proteins or Casamino acids with B vitamins to rice media stimulate the growth of *B. cereus* (Morita and Woodburn, 1977). Serological identification of the emetic antigen has not been possible because of its low molecular weight, so biological assays are used. Rice mixtures containing *B. cereus* emetic toxin fed to monkeys cause emesis in 50% of the animals (Melling et al., 1976). Intravenous injection of kittens with concentrated culture fluids produced by culturing *B. cereus* in BHI broth supplemented with 0.1% glucose (pH = 7.4) and heating at 100°C for 1.5 h has caused emesis (Bennett and Harmon, 1988).

Other *Bacillus* Infections

Other nonanthrax *Bacillus* infections have been classified as 1) local, usually involving the eye or an isolated organ that was previously damaged; 2) deep tissue or mixed, in which a *Bacillus* species is usually found in the company of other organisms; and 3) disseminated, in which the organism is consistently cultured from the blood or cerebrospinal fluid of a seriously ill person (Farrar, 1963).

Local Infections

The eye has been the organ most commonly infected by nonanthrax *Bacillus* species, especially *B. cereus*. They can cause conjunctivitis, keratitis, iridocyclitis, dacryocystitis, orbital abscess, and panophthalmitis. *Bacillus* species are most commonly isolated in the setting of penetrating nonsurgical trauma. In a review of nonsurgical post-traumatic endophthalmitis, *Bacillus* species ranked as the second most common pathogen in five of six series reviewed (Davey and Tauber, 1987). An intra-ocular foreign body such as a metal projectile is often present in this setting or the injury occurs in a rural or farm location where there is a greater risk of contamination with dust or soil. *B. cereus* is one of the most destructive organisms to infect the eye (Davey and Tauber, 1987). Panophthalmitis with *Bacillus* species also occurs in the setting of hematogenous dissemination, as in intravenous drug abusers (Davey and Tauber, 1987; Pearson, 1970; Young et al., 1980).

These patients present with a fulminant endophthalmitis or panophthalmitis that usually results in blindness and the need for enucleation. Although the exact pathogenesis is ill-defined, it is probably related to endogenous infection from contaminated drugs and paraphernalia (Shamsuddin et al., 1982). Approximately 50% of cultures of heroin and drug paraphernalia contain *Bacillus* species, making them the most common organism isolated (Tuazon et al., 1974). In one patient with panophthalmitis, *B. cereus* was isolated from a vitreous aspirate and also from the syringe used for injection (Young et al., 1980). Severe suppurative endogenous panophthalmitis caused by *B. cereus* has also resulted from injection of vitamin B (Bouza et al., 1979) and also after a blood transfusion (Kerkenezov, 1953).

Clinical features are fairly characteristic, with intense pain followed by chemosis, periorbital swelling, and severe proptosis. Within 48 h, a corneal ring abscess develops. This is a hallmark of an intraocular infection with *Bacillus* organisms. The end result is irreversible loss of vision and eventual loss of the eye (Davey and Tauber, 1987; O'Day et al., 1981). A characteristic feature of infection with *B. cereus* is its frequent association with fever and leukocytosis, which are not usually seen in panophthalmitis caused by other bacteria (Davey and Tauber, 1987). Early diagnosis is important. This organism should be considered whenever ocular infection occurs in the setting of penetrating injury with probable soil contamination or in an intravenous drug user. Vitreous aspiration with Gram stain and culture is diagnostic.

Empiric therapy should begin before isolation of an organism and the availability of antibiotic susceptibility data. For treatment of severe ocular infections, early vitrectomy and intravitreal antibiotics, combined with systemic and periocular antibiotics, appear to offer the best chance for success (Davey and Tauber, 1987). Clindamycin or vancomycin should be combined with an aminoglycoside.

The first case of human infection with *B. thuringiensis* was reported by Samples and Buettner (1983) in a healthy farmer who splashed an insecticide containing the organism into his eye. The organism was recovered from a corneal ulcer. The clinical course was much less fulminant than with *B. cereus* infection.

Mixed Infections or Deep-Tissue Infections

This category includes cellulitis, traumatic wound infections, surgical wound infections, infected burns, infected necrotic tumors, necrotizing fasciitis, pyelonephritis, pericarditis, and pneumonia (Bias, 1927; Farrar, 1963; Ihde and Armstrong, 1973; Logan et al., 1985b; Pearson,

1970; Pennington et al., 1976; Tuazon et al., 1979). *Bacillus* species are rare pulmonary pathogens. *B. cereus* has caused pneumonia in patients with malignancy (Bekemeyer and Zimmerman, 1985; Pennington et al., 1976) and in those with no known immune compromise (Jonsson et al., 1983). Cavitating pneumonia has been reported (Leff et al., 1977). *B. sphaericus* caused a large pseudotumor of the lung in a patient with chronic asthma on corticosteroids (Isaacson et al., 1976). Symptoms are indistinguishable from other bacterial pneumonias. Complications include empyema, massive hemoptysis, acute respiratory failure, tension pneumothorax, and bronchopleural fistula (Bekemeyer and Zimmerman, 1985). Cases of necrotizing pneumonia and empyema may require resection of infected lung and decortication (Jonsson et al., 1983).

Bacillus species, especially *B. cereus*, can infect traumatic wounds in both the normal host and the immunocompromised host (Dryden and Kramer, 1987; Jaruratanasirikul et al., 1987). In the immunocompromised patient, the trauma can be as minor as scratch marks exposed to muddy water (Jaruratanasirikul et al., 1987). Severe toxicity may develop when *B. cereus* infects relatively minor burns (Attwood and Evans, 1983). In addition to traumatic wounds, *B. cereus* has infected breast implants (Sliman et al., 1987) and pin sites following pin placement and plaster fixation of an open forearm fracture (Rutala et al., 1986). In the later situation, *B. cereus* was recovered from plaster-impregnated gauze rolls and tapwater samples. The pins may have facilitated infection by serving as a conduit for the plaster-associated bacteria to the pin insertion sites. Plaster samples were subsequently sterilized by steam or gas.

Primary cutaneous *B. cereus* infection was recently reported in neutropenic children (Henrickson et al., 1989). In these cases, there was a spring and summer seasonal predominance but no history or signs of injury to the skin. Vesicles or pustules with rapidly spreading cellulitis were seen only on the extremities. Although neutropenic and febrile, the children were not otherwise systemically ill. Cultures of vesicle fluid or wound drainage yielded pure growth of *B. cereus*. All cases responded to antibiotic therapy. Cutaneous *B. cereus* infection is clinically similar to cutaneous anthrax and has the same seasonality. This entity may be related to the secretion of the same or similar exotoxins.

Necrotizing fasciitis caused by *Bacillus* species has been reported in a leukemic patient and in a patient with sickle cell disease (Sliman et al., 1987; Tuazon et al., 1979). In both cases, *Bacillus* species grew in pure culture from deep tissue. *B. cereus* has been associated with myonecrosis postoperatively (Fitzpatrick et al., 1979) and fol-

lowing trauma (Johnson et al., 1984; Groschel et al., 1976). Treatment of these deep soft tissue infections requires surgical debridement and systemic antibiotics. Amputation may be necessary.

Streptococcal and other organisms are frequently isolated along with the *Bacillus* species. It is frequently unclear what role the *Bacillus* plays in these mixed infections. It may be a copathogen, a secondary invader, or a colonizer. Ihde and Armstrong (1973) found that whether the *Bacillus* was treated did not affect outcome in surgical wounds. However, they did note a change in the character of the drainage from sanguinous to serous after the *Bacillus* was no longer recovered in culture.

Disseminated Infections

The category of disseminated infections includes bacteremia, endocarditis, meningitis, and other such infections. Since *Bacillus* species are ubiquitous in the environment, it is not suprising that positive cultures for these organisms are fairly common. They are only rarely associated with actual infection, however. The prevalence of positive blood cultures for *Bacillus* species has ranged from 0.1 to 0.9% (Dalton and Allison, 1967; Kotin, 1952; Pearson, 1970). Isolation of *Bacillus* species from blood is clinically significant in 5 to 10% of cases (Weber et al., 1989).

Bacteremia is relatively common in *Bacillus* infections and the incidence is increasing. *B. cereus* is the predominant species. Bacteremia can be a complication of indwelling intravascular catheters (Banerjee et al., 1988; Cotton et al., 1987; Sliman et al., 1987). Scanning and transmission electron microscopy of a Hickman catheter after removal showed *Bacillus* organisms embedded in a biofilm composed of Gram-positive cocci and glycocalyx (Banerjee et al., 1988). In a study by Sliman et al. 1987, an intravascular device proved to be the source of the bacteremia in one-half of the cases, as evidenced by a positive culture of purulent drainage from a peripheral vein or the recovery of greater than 15 colonies from a semiquantitative culture of a catheter tip. Isolated bacteremias are usually eradicated easily. Bacteremia may clear after removal of an intravascular device in the absence of specific antibacterial therapy (Sliman et al., 1987). For *Bacillus* bacteremias in immunocompromised patients (especially those who are neutropenic) the catheter should be removed and systemic antibiotics administered (Banerjee et al., 1988; Cotton et al., 1987).

Bacteremia has also occurred in association with infections in a variety of body sites. Unlike those with primary bacteremia, patients with infections of solid organs frequently have high morbidity. Some of these cases of bacteremia may actually have been cases of endocarditis, and some were treated as such. A relatively common situation has been pneumonia with bacteremia in individuals with acute leukemia and neutropenia (Coonrod et al., 1971a; Ihde and Armstrong, 1973; Pennington et al., 1976; Sathmary, 1958). The neutropenic patient is at risk for life-threatening bacteremia following seemingly minor trauma (Guiot et al., 1986).

An outbreak of five cases of bacteremia occurred in a group of hemodialysis patients exposed to contaminated dialysis fluid and equipment (Curtis et al., 1967). Individual cases of bacteremia in association with volvulus and peritonitis (Sugar and McCloskey, 1977), infected ventriculojugular shunt cured only by removal of the shunt (Cox et al., 1959), and sepsis in a newborn infant, perhaps related to contaminated blood transfusion (Yow et al., 1949), have been reported.

There have been five outbreaks of pseudobacteremia associated with contaminated broth culture media (Crowley et al., 1983; Noble and Reeves, 1974), contaminated syringes (MacDonald, 1982) or alcohol swabs (Berger, 1983), and a contaminated needle in a radiometric blood culture analyzer (Gurevich et al., 1984).

Endocarditis due to *Bacillus* organisms is uncommon; only about 12 cases have been reported in the English-language literature (Agarwala et al., 1975; Block et al., 1978; Farrar, 1963; Oster and Kong, 1982; Yeh et al., 1967). More than one-half the cases have occurred in intravenous drug users (Craig et al., 1974; Reller, 1973; Tuazon et al., 1979; Weller et al., 1979), probably related to the frequent presence of *Bacillus* organisms on injection paraphernalia and in street heroin (Tuazon et al., 1974). *Bacillus* endocarditis cannot be distinguished from other bacterial causes of endocarditis on the basis of clinical features. The majority of patients have had low-grade fever and heart murmurs. There is a predominance of tricuspid valve involvement. Some have had hepatosplenomegaly, and a few have had peripheral stigmata. *B. cereus* has caused infection of a permanent ventricular pacemaker (Sliman et al., 1987). Echocardiogram revealed a vegetation on the pacemaker wire in the right ventricle. The organism was isolated from blood, the subcutaneous pacemaker pouch, and right ventricular tissue. There was persistence of the organism despite 10 days of appropriate antibiotic therapy. Cure was achieved only after removal of the pacemaker and 4 weeks of antibiotic therapy postoperatively.

In a study of 849 cerebrospinal fluid (CSF) cultures, nine were positive for nonanthrax *Bacillus* species. In seven patients the isolates represented contamination (Feder et al., 1988).

There has been at least one outbreak of pseudo-meningitis caused by contamination of broth culture media by *Bacillus* species (Lettau et al., 1988). True meningitis due to *Bacillus* organisms has been reported primarily in situations in which there is either direct access of exogenous organisms to the subarachnoid space (spinal anesthesia, head trauma, and neurosurgical procedures with and without foreign bodies), or in individuals whose immune function is depressed (newborn state, alcoholism, and hematologic malignancy), although at least one case has been reported in an otherwise healthy individual (Allen and Wilkinson, 1969; Boyette and Rights, 1952; Colpin et al., 1981; Farrar, 1963; Park et al., 1976; Patrick et al., 1989; Weinstein and Colburn, 1950; Weidermann, 1987). It has also occurred secondary to other infections such as otitis, mastoiditis, infected subdural hematoma, and hematogenous dissemination from a urinary tract infection. Causative organisms have included *B. alvei*, *B. megaterium*, *B. subtilis*, *B. pumilus*, *B. circulans*, *B. sphaericus*, and *B. cereus*. Single cases of postoperative ventriculitis due to *B. licheniformis* and *B. cereus* have been reported (Young et al., 1982). The latter infection was in a patient with a ventriculoperitoneal shunt. Removal of any foreign body is essential for cure.

Brain abscess with *B. cereus* has been reported rarely (Ihde and Armstrong, 1973; Jenson et al., 1989). All of the patients were immunocompromised. In two of the patients, involvement of the brain parenchyma was probably via the hematogenous route from primary foci of *B. cereus* pneumonia (Ihde and Armstrong, 1973). Diagnosis was made postmortem. The other patient had multiple brain abscesses, concurrent meningitis and may have acquired the infection hematogenously from a potentially contaminated transfusion (Jenson et al., 1989). He was successfully treated with vancomycin and gentamicin, and vancomycin and rifampin for a total of 8 weeks. Mild memory deficit and mild residual paresis of the right upper extremity persisted as sequelae of the infection.

Acute and chronic osteomyelitis and septic arthritis caused by *Bacillus* species are rare. In a review of *Bacillus* infections, 10 cases of bone and joint infection were reported (Pearson, 1970). The organism was present in mixed culture in 9 of the 10 cases, and in 5 it was obtained from a wound or drainage. Most of the patients had previous trauma. Acute vertebral osteomyelitis caused by *B. cereus* has been reported in an intravenous drug user (Tuazon et al., 1979). An association between *Bacillus* species osteomyelitis and sickle cell disease has been noted (Reboli et al., 1989; Sliman et al., 1987; Solny et al., 1977). *B. alvei* caused infection of a prosthetic hip in a patient with sickle cell disease, probably via the hematogenous route (Reboli et al., 1989). In general, bacteremia in the setting of osteomyelitis is rare. *B. cereus* septic arthritis has occurred as a complication of arthrography (Robinson, 1979). *Bacillus* infections of bone and joints have been difficult to eradicate, require multiple surgical procedures, and cause substantial morbidity (Sliman et al., 1987).

Except for the characteristic signs and symptoms of panophthalmitis, most infections due to nonanthrax *Bacillus* species do not have distinctive clinical presentations, and although serious infections are being recognized with increasing frequency, endocarditis, meningitis, and sepsis are still sufficiently rare that the question of contaminant vs. pathogen is still valid. Diagnosis of true infection rests on the isolation of the organism from blood, normally sterile body fluids, or closed spaces (such as the pleural space) of a susceptible host in the appropriate clinical setting. A positive Gram stain and growth in pure culture are contributory evidence of the *Bacillus* being a true pathogen, whereas growth only in thioglycolate broth (indicating a small number of organisms) or the presence of *Bacillus* organisms in mixed culture with other species is suggestive of a contaminant. Although infection can occur in healthy persons, it is most often seen in immunocompromised patients such as those with hematologic malignancies, intravenous drug users, or patients with severe trauma (Ihde and Armstrong, 1973; Pennington et al., 1976; Tuazon et al., 1979).

Hypersensitivity Reactions

Allergic reactions have been described in workers exposed to autolysates of *B. subtilis* used as enzymes in laundry products (Dubos, 1971; Flindt, 1969; Greenberg et al., 1970; Pepys et al., 1969). These reactions were mainly dermatitis and asthma. Those involved showed immediate and late reactions to inhalation and skin prick tests (Flindt, 1969). Hypersensitivity pneumonitis following exposure to wood dust contaminated with *B. subtilis* has been described (Johnson et al., 1980.)

Treatment and Prognosis

Many strains of *Bacillus* species are susceptible to the aminoglycosides, tetracycline, chloramphenicol, erythromycin, vancomycin, and clindamycin (Coonrod et al., 1971b; Tuazon et al., 1979). Susceptibility to penicillin, ampicillin, methicillin, and cephalosporins is usually species related and has been shown to be high for *B. subtilis* and low for *B. cereus*. *B. pumilus* is intermediate between *B. subtilis* and *B. cereus*. *B.*

cereus is the most resistant species, showing resistance to the penicillins and cephalosporins that may be attributable to its ability to produce a β-lactamase that hydrolyzes these agents (Sabath and Abraham, 1965). In vitro susceptibility tests reveal all *B. cereus* strains to be susceptible to imipenem, vancomycin, chloramphenicol, gentamicin, and ciprofloxacin (Weber et al., 1988). Non-*B. cereus* strains were most susceptible to imipenem, vancomycin, daptomycin, and ciprofloxacin (Weber et al., 1988). Further studies are needed before imipenem and ciprofloxacin can be recommended for treatment of *Bacillus* infections. Clindamycin or vancomycin with or without an aminoglycoside may be the treatment of choice for endocarditis or sepsis with *B. cereus* (Sliman et al., 1987; Tuazon et al., 1979). Other species can be treated with penicillins or cephalosporins if they are found to be susceptible. Surgical drainage may be necessary in closed space and soft tissue infections. Infected prosthetic heart valves may need replacement but infected native valves usually can be treated with antibiotics alone.

Morbidity and mortality for disseminated infections were high prior to the antibiotic era. Except for panophthalmitis, which usually results in loss of vision and of the eye, other infections by *Bacillus* species have a good prognosis unless the underlying disease (such as malignancy) supervenes. *B. cereus* endocarditis in intravenous drug users has a relatively benign course and favorable prognosis. Of patients with meningitis, approximately 40% survived; however, most have had central nervous system sequelae.

Pathology and Pathogenesis

The pathologic changes in organs infected by nonanthrax *Bacillus* species are the inflammatory reactions caused by bacteria in general. There have not been any special or pathognomonic changes described in humans. Subcutaneous injection of *B. cereus* in mice, guinea pigs, and rabbits has caused deep, hemorrhagic, ulcerative skin lesions and death (Burdon et al., 1967). Direct cytotoxicity of *B. cereus* culture filtrate for tissue culture cells has been noted (Bonventre, 1965). *B. cereus* culture filtrates were noted to kill mice and rats within minutes after intravenous injection (Bonventre and Eckert, 1963). Pathologically there were widespread thromboses. The filtrates were noted to be hemolytic and dermonecrotic. The pathologic changes included focal areas of coagulative necrosis and inflammatory cells in the pulmonary arterioles and capillaries. Thrombi were found in the pulmonary vessels. Because no organisms were seen in tissue sections, toxins were believed to be responsible for illness and death. This hypothesis was further supported by the finding that antitoxic serum had a protective effect.

In one case of neonatal meningitis, postmortem histopathologic examination revealed an invasive disease involving the brain parenchyma. The cellular composition of the CSF was significant for an increased number of mononuclear cells, implying a chronic infection (Patrick et al., 1989). The invasive and destructive nature of *B. cereus* has been demonstrated by microscopic examination of brain tissue in a patient with multiple brain abscesses (Jenson et al., 1989). There was extensive bacterial invasion of brain tissue and hemorrhagic necrosis. Localized tissue infection with *B. cereus* is usually necrotizing due to the production of a potent exotoxin and usually results in rapid, fulminant tissue destruction.

Different *Bacillus* species produce a variety of enzymes including amylase, collagenase, hemolysin, lecithinase, phospholipase, protease and urease, antimicrobial substances (bacitracin, gramicidin, polymyxin, and tyrocidine), pigments, and toxins (Williams, 1981). *B. cereus* produces distinct extracellular products: β-lactamases, hemolysins, phospholipase C, and two lethal toxins (Coolbaugh and Williams, 1978; Gilbert and Kramer, 1984; Thompson et al., 1984; Turnbull et al., 1979a; Turnbull, 1981). The lethal toxins may contribute to the virulence of *B. cereus*. One has a molecular weight of approximately 50,000 and is a "loop fluid-inducing/skin test/necrotic toxin." The other toxin has a molecular weight of 55,000 to 56,000 and is cereolysin. It interacts with cholesterol as a receptor in the host cell membrane. It demonstrates vascular permeability activity which is difficult to distinguish from that of "loop fluid-inducing/skin test/necrotic toxin." Two hemolysins have been described. One has a molecular weight of 52,000 and causes immediate lysis of red blood cells (Coolbaugh and Williams, 1978). The other has a molecular weight of 31,000. *B. cereus* is not the only species that produces hemolysins; at least 18 *Bacillus* species have the capability (Bernheimer and Grushoff, 1967).

The lysins of some species resemble streptolysin O. Paper chromatography has demonstrated that hemolysins are not the same as phospholipases (Stein and Logan, 1965). Phospholipases and lecithinases may be identical, however. Phosphatidyl-choline hydrolase is also known as phospholipase C. It may have a role in ocular infections by disrupting cell membrane phospholipids after they have been exposed by the action of other toxins (Turnbull, 1981). Hemolysins may be responsible for changes in quality of drainage from infected wounds (Ihde and Armstrong, 1973).

Specimen Collection, Transport, Maintenance, and Microscopic Examination

Specimens and cultures which may contain *B. anthracis* require specific laboratory safety precautions. All work should be performed in a biological safety hood since aerosolization may carry a risk of infection by inhalation. Personnel should wear gloves while processing specimens and performing tests. Bench space should be disinfected with 1% sodium hypochlorite after work is completed and contaminated items should be autoclaved prior to disposal.

Vesicular fluid or exudate from cutaneous lesions suspected of being infected with *B. anthracis* should be sampled using dry sterile swabs (Doyle et al., 1985). Blood cultures should be taken and specimens of lymph nodes, spleen, peritoneal exudate, or other tissues obtained by biopsy or necropsy should be obtained for microscopic examination and culture. Specimens should be placed in secure sterile containers for transportation to the laboratory. The swabs can be used for inoculation of media for cultivation of the organism and for preparation of smears. Smears should be fixed for 3 to 5 min in Zenker's solution (2.5% aqueous potassium bichromate and 8% mercuric chloride) or 10% Formalin (37% formaldehyde) for 10 min to ensure inactivation of spores; even repeated passage of the slide through a flame may not kill all spores. Smears can be stained with either Gram or Giemsa stain. Microscopically, *B. anthracis* appears as a large Gram-positive rod with square ends, 1.0 to 1.3 μm wide and 3 to 10 μm long. Spores when present are oval, located centrally or subterminally, and produce little or no swelling of the cell. Capsules may often be seen in direct smears from infected tissues using capsule-staining methods, and specific fluorescent monoclonal antibody reagents may be obtained from the CDC, Atlanta, GA.

Clinical specimens such as blood, cerebrospinal fluid, and wound exudates for isolation of other *Bacillus* species do not require special handling. Prompt plating to prevent growth of contaminants is necessary. Tissue specimens should be macerated and inoculated into a peptone broth. As spores are viable for long periods, maintenance of cultures after initial isolation is usually not difficult. Preservation in sterile soil has been suggested, but nutrient agar with manganese sulfate is equally good for sporulation and maintenance and is more convenient than soil extract (Norris et al., 1981). *Bacillus* species may be stored on agar slants, frozen at −70°C, or freeze-dried for long-term maintenance. Repeated freezing and thawing should be avoided. Freeze-drying may alter the character of the organisms as can repeated subculturing at frequent intervals.

In cases of *B. cereus* food poisoning, food stuffs to be examined should be kept at or near 5°C until examined. They can be stored in a refrigerator for up to 48 h. If they are to be stored for longer periods of time they should be frozen. Twenty-five-gram portions of food are homogenized in Butterfield's buffer in a blender and diluted before testing (Bennett and Harmon, 1988).

Isolation and Identification

Cells of *Bacillus* organisms range from approximately 0.4 to 2 μm in width and from 3 to 12 μm in length. They are straight or slightly curved and have round or square ends. An initial Gram stain should be done on any suitable clinical material. It is the most rapid and easiest way to assess the quality of the sample and to have a preliminary categorization of the pathogen. If Gram-positive bacilli are seen, further testing will be needed to distinguish *Bacillus* species from other Gram-positive rods such as *Kurthia*, *Listeria*, *Rothia*, *Corynebacterium*, *Erysipelothrix*, and *Clostridium*. *Bacillus* species may at times resemble Gram-negative bacilli, especially cells from colonies that are older. In contrast to the nonfermenting Gram-negative rods, most *Bacillus* species do not usually grow on enteric agars. In addition, *Bacillus* species are susceptible to vancomycin, whereas Gram-negative nonfermenters are not, and a KOH test can be performed in which Gram-negative rods show a viscous thread (Gregersen, 1978). A colony is stirred into one or two drops of 3% KOH on a glass slide with a loop and the loop is slowly raised. Gram-negative bacteria make the KOH viscous, and a thread of this material follows the loop for 0.5 to 2 cm or more. Some *Bacillus* strains, particularly cells from older cultures that have lost their Gram positivity, may give a Gram-negative-type reaction; this test is of value for identification of *Bacillus* species only if no viscous thread is formed.

B. anthracis produces large, raised, opaque, grayish, granular colonies within 24 h when grown on ordinary laboratory media at 37°C. The borders of the colonies are irregular and may resemble a "Medusa head" or "comet tails." The cells adhere tenaciously to one another and if material is picked from the colony with an inoculating needle the remainder of the colony may assume a "stalagmite" shape.

Doyle et al. (1985) have provided a general approach for the identification of *B. anthracis*. In order of importance, they recommend seven procedures:

1. Culture the specimen on sheep blood agar. If the specimen is heavily contaminated use the selective PLET medium of Knisely, which contains polymyxin, lysozyme, EDTA, and thallous acetate. Each batch of this medium should be tested to demonstrate that it will allow growth of known strains of *B. anthracis*, while inhibiting growth of *B. cereus*.

2. Subculture the isolate on nutrient agar or BHI agar containing 0.5% sodium bicarbonate and supplemented with 0.7% bovine serum albumin, and incubated in 5% CO_2. Demonstrate the presence of a capsule by using either M'Fadyean stain or fluorescein-labelled anti-poly-D-glutamic acid. Fluorescent-antibody reagent may be used to detect organisms in tissue sections or from blood smears or culture. Fluorescein conjugates of heterologous sera and monoclonal antibody to capsule can be obtained from the CDC, Atlanta.

3. Determine the toxin-antitoxin reaction using a double diffusion assay with R-medium plus 1.5% agar (Ristroph and Ivins, 1983). A positive reaction confirms identification of *B. anthracis* but a negative reaction does not exclude it.

4. Test for susceptibility to penicillin; *B. anthracis* is the only *Bacillus* species which is consistently susceptible to penicillin G.

5. Determine the rates of hydrolysis of p-nitro-phenyl-α-D-glucopyranoside and p-nitro-phenyl-α-D-maltoside in the presence and absence of 1% Triton X-100. Alpha-glucosidase activity is increased in *B. anthracis* by incubation in the detergent; glucosidase activity in other members of the *B. cereus* group is diminished.

6. Test for agglutination of cells by soybean *(Glycine max)* lectin but not by the snail *Helix pomatia* lectin.

7. Determine susceptibility to gamma phage (Doyle et al., 1985; Rees et al., 1988).

The "string of pearls" test is based upon the susceptibility of *B. anthracis* to penicillin (Rees et al., 1988). A drop of young (12–18 h) broth culture is placed on two plates of nutrient agar that contains either 0.5 or 10 units of penicillin per milliliter. After incubating the inoculated media at 37° for 3 to 6 h, a cover slip is placed directly on the growth present on the medium. Microscopic examination of the underlying growth under low magnification reveals the presence of large rounded cells (or a "string of pearls"). Little or no growth should be seen on the medium containing 10 units of penicillin per milliliter. Approximately 95% of strains of *B. anthracis* give a positive test; only 8% of non-anthrax *Bacillus* strains are positive.

It is fairly easy to differentiate between *B. anthracis* and other *Bacillus* species with the exception of *B. cereus*. Table 1 shows important differential characteristics between *B. anthracis* and other pseudoanthrax bacilli. Unlike *B. anthracis*, *B. cereus* is usually resistant to penicillin, is resistent to gamma phage, does not encapsulate on bicarbonate agar, and does not show fluorescence of cell wall and capsule on fluorescent antibody-stained smears.

Bacillus cereus is a large-celled member of group 1. It has a cell width greater than 0.9 μm and spores which do not appreciably swell the sporangium. It is controversial whether *B. anthracis*, *B. mycoides*, and *B. thuringiensis* should have species status or be considered variant species of *B. cereus*. Within this species, strain heterogeneity is relatively common but typical characteristics are very stable. Four

Table 1. Differences between *Bacillus anthracis* and the pseudoanthrax bacilli.

B. anthracis	Anthrax-like or pseudoanthrax bacilli
Nonmotile	Generally motile[a]
Capsulated	Noncapsulated[a]
Grows in long chains	Grow in short chains
No turbidity or pellicle in broth	Often turbidity and pellicle in broth
No growth on penicillin agar (10μg/ml)	Usually good growth on penicillin agar
Inverted fir-tree growth in gelatin	Fir-tree growth absent or atypical[a]
Methylene blue reduced weakly	Methylene blue usually reduced strongly
Hemolysis of sheep cells weak	Hemolysis of sheep cells often strong
Liquefaction of gelatin slow	Liquefaction of gelatin usually rapid[a]
Lecithinase reaction weakly positive	Lecithinase reaction strongly positive[a]
Ferments salicin slowly	Often ferment salicin rapidly
Polysaccharide precipitin reaction strongly positive	Polysaccharide precipitin reaction weakly positive
Produces toxin, neutralized by *B. anthracis* antitoxin	Any toxic substances produced not neutralized by *B anthracis* antitoxin
Pathogenic to laboratory animals	Mostly nonpathogenic. If pathogenic, produce disease unlike anthrax
Susceptible to phage	Insusceptible to phage
Culture filtrates nontoxic to tissue culture cells	Culture filtrates toxic to tissue culture cells[a]

[a]For *B. cereus*. From Reboli and Farrar (1988).

characteristics are useful in differentiating members of the *B. cereus* group (hemolytic activity, motility, rhizoid growth, and detection of toxin crystals) (Harmon, 1982).

Hemolytic Activity

Tryptic-soy-sheep blood agar is inoculated by lightly touching the agar surface with a loopful of culture. The plate is incubated at 30°C for 24 h and observed for hemolytic activity as indicated by a zone of complete hemolysis around the growth. *B. anthracis* is nonhemolytic; *B. cereus* is strongly hemolytic. *B. mycoides* and *B. thuringiensis* are weakly hemolytic or produce hemolysis only under the growth.

Motility Tests

Motility may be determined by direct microscopic examination or by using semisolid *B. cereus* motility medium (Harmon, 1982). The medium is inoculated by stabbing down the center and is incubated at 30°C for 18 to 24 h. Motile strains produce diffuse growth into the medium away from the stab; nonmotile strains except for *B. mycoides* grow only in and along the stab. Questionable results should be confirmed by the microscopic motility test which is performed by adding 0.2 ml of sterile distilled water to a nutrient agar slant, inoculating the slant with a loopful of culture, and incubating at 30°C for 6 to 8 h. A loopful of liquid culture from the base of the slant is suspended in a drop of water on a clean slide, covered with a cover slip, and examined immediately for motility (Bennett and Harmon, 1988). *B. cereus* and *B. thuringiensis* are motile; *B. anthracis* and the rhizoid strains of *B. mycoides* are nonmotile.

Rhizoid Growth

A pre-dried nutrient agar plate is inoculated centrally and incubated at 30°C for 1 to 2 days. If the culture is rhizoid, root-like structures will develop up to several centimeters from the point of inoculation. Rhizoid growth is characteristic only of *B. mycoides*.

Detection of Toxin Crystals

Endotoxin crystals of *B. thuringiensis* may be detected by phase contrast microscopy or by staining with 0.5% aqueous basic fuchsin or TB carbol fuchsin. The other members of the *B. cereus* group do not produce endotoxin crystals that can be detected by staining.

In certain clinical situations in which *Bacillus* infection is suspected, specimens can be suspended in distilled water or nonnutrient buffer

and pasteurized (heated at 70–80°C for approximately 10 min) to kill contaminating vegetative cells. Spores will survive. Caution is needed, however, as organisms may be present only in the vegetative state and no growth will be achieved. In addition, heat treatment of spores may cause mutations. Air-drying and treatment with 50% ethanol for 1 h are also effective for selecting out sporulating organisms. Treatment with ethanol may be more desirable. Sporulation is often stimulated on esculin agar and may be facilitated on an acidified medium such as triple sugar iron agar (TSI).

Isolation is relatively easy, as most medically important *Bacillus* species grow well on blood agar or simple nutrient agar, which is composed of a meat extract in peptone with a pH of 6.8 to 7.0. Growth is sometimes improved by glucose but not by serum. Cultures should be incubated aerobically at 35 to 37°C for 24 h. Some strains require enrichment techniques using specific broth, incubation under specific conditions of pH, temperature, and salt concentration, or plating on specific agar media. Colony morphology and microscopic appearance form the basis of preliminary identification.

Nutrient Agar (pH = 6.8–7.0)

Agar	15.0 g
Peptone	5.0 g
Meat extract	3.0 g
Distilled water	To 1 liter

For sporulation, add 5 mg of hydrous manganese sulfate per 1 liter of medium.

Nutrient Broth (pH = 6.8)

Peptone	5.0 g
Meat extract	3.0 g
Distilled water	To 1 liter

Colony morphology should be observed from young (18–24 h) cultures grown on nutrient agar under aerobic conditions. Single colonies are usually two to several mm in diameter and may have a finely granular appearance. Other colonies appear membranous and wrinkled (Wilson, 1983). *B. cereus* colonies vary from small, shiny, and compact to large, feathery, and spreading. On sheep blood agar, a lavender-colored colony with beta hemolysis is seen. Colonies of *B. subtilis* are usually large, dull, and flat, with a ground glass appearance. The microscopic appearance of cells is best observed on slides freshly coated with a thin layer of 2% water agar and examined with a phase-contrast microscope (Claus and Berkeley, 1986). Colonies suspected of being *B. cereus* should be subcultured and checked for motility, hemolysis, and susceptibility to penicillin. Serotyping with antibody directed against flagellar antigens should be employed for confirmation of *B. cereus*.

Four selective and differential plating media have been described for the isolation of *B. cereus* (Holbrook and Anderson, 1980; Kim and Goepfert, 1971; Mossell et al., 1967; Szabo et al., 1984). Three of them contain mannitol to enhance differentiation. The most common procedure for isolation and presumptive identification of *B. cereus* is based on the egg yolk reaction (Doyle et al., 1985). In this reaction, turbidity develops in egg yolk or in agar that contains egg yolk. It is caused by an extracellular substance (or substances) referred to as egg yolk turbidity factor, lecithinase, or phospholipase. The reaction may be due to a more complex series of events than the action of a single enzyme (Kushner, 1957). Material to be cultured is placed onto blood agar (Harmon et al., 1984; Schiemann, 1978) containing polymyxin to suppress Gram-negative growth. Plates are incubated at 37°C overnight, and suspect colonies are then inoculated onto mannitol egg yolk agar by stabbing various sites on the plate and incubated at 35 to 37°C overnight. As *B. cereus* is lecithinase positive and does not ferment mannitol, plates are examined for a zone of precipitation or halos around sites of inoculation and for lack of evidence of fermentation. (If fermentation has occurred, the background color changes from violet to yellow.) Similar reactions may be seen with other members of the *B. cereus* group (*B. anthracis*, *B. thuringiensis*, and *B. mycoides*). Some strains of *B. cereus* give a weak or negative egg yolk reaction. On polymyxin pyruvate-egg-yolk-mannitol-bromothymol blue-agar (PEMBA) media, *B. cereus* has a distinct colonial appearance (Holbrook and Anderson, 1980). After incubation at 37°C for 24 to 48 h, colonies of *B. cereus* are rough, dry, and crenated or rhizoid. They have a distinctive bright blue color. (*B. thuringiensis* has the same appearance).

Egg-Yolk Reaction Medium

Tryptone	10 g
Disodium hydrogen phosphate	5 g
Potassium dihydrogen phosphate	1 g
Sodium chloride	2 g
Magnesium sulfate	0.1 g
Glucose	2 g
Distilled water	To 1 liter

Egg yolk (1.5 ml) is added to 100 ml of basal medium. The medium is held refrigerated overnight and the supernatant broth is aliquoted.

Measuring the Egg-Yolk Reaction
Cultures are inoculated into egg-yolk broth and into a control broth without egg yolk. After incubation for 1, 3, 5, and 7 days observe for the appearance of a heavy white precipitate in or on the surface of the egg-yolk medium.

PEMBA (Oxoid) (pH = 7.2) (Holbrook and Anderson, 1980)

Agar	18.0 g
Peptone	1.0 g
D-Mannitol	10.0 g
$MgSO_4$	0.1 g
NaCl	2.0 g
Na_2HPO_4	2.5 g
KH_2PO_4	0.25 g
Bromothymol blue	0.12 g
Distilled water	To 1 liter

Prior to use, the following are added to 90-ml aliquots of the basal medium:

20% (w/v) Sodium pyruvate	5 ml
Polymyxin	100 units/ml
Egg-yolk emulsion (Oxoid)	5 ml

Surface plating is usually employed to determine the presence of *B. cereus* in outbreak foods. The official method of the Association of Official Analytical Chemists (AOAC) for enumerating *B. cereus* in foods uses mannitol-egg yolk-polymyxin (MYP) media (Lancette and Harmon, 1980). Blood agar, PEMBA, and KG agar (Kim and Goepfert, 1971) are of similar efficacy and are used in some laboratories. If *B. cereus* counts are expected to be less than 1,000 per gram, the alternative AOAC most probable number (MPN) method using tryptic-soy-polymyxin broth is recommended (Lancette and Harmon, 1980). Presumptive colonies from the above plating media should be transferred to nutrient agar slants and subjected to biochemical tests.

The nature of members of this genus is such that strains may not exhibit the important characteristics of the genus, let alone the species. Because of the number of species and variant strains, it may not be possible to speciate quickly. Norris et al. (1981) suggest basing species identification on the results of two morphologic features, width of the rod, and parasporal body in the sporangium, and 11 tests: catalase production, Voges-Proskauer reaction, pH in Voges-Proskauer broth, growth in anaerobic agar, growth at 50°C, growth at 65°C, growth in 7% sodium chloride, production of acid and gas from glucose, reduction of nitrate to nitrite, hydrolysis of starch, and casein decomposition. Citrate utilization may not be useful because of disagreement in interpretation. Wilson (1983) advocates also testing for gelatin liquefaction, urease production, and growth in 4% sodium chloride. A scheme for identification of *Bacillus* species is provided in Table 2. Media recipes and performance of tests are based on the work of Gordon et al. (1973) and Smith et al. (1952).

Catalase Test

Cultures grown for 24 to 48 h on nutrient agar slants are flooded with 0.5 ml of 10% hydrogen peroxide. The reaction is positive if there is a rapid bubbling of gas. If

Table 2. Characteristics useful to differentiate among some species of *Bacillus*.

Bacillus species	Rods 1.0μm wide or wider	Parasporal bodies	Growth in anaerobic agar	Growth at 50°C	Growth at 65°C	Growth in 4% NaCl	Growth in 7% NaCl	Catalase	Voges-Proskauer reaction	Acid from glucose	Acid and gas in glucose	NO_3 reduced to NO_2	Starch hydrolyzed	pH in V-P medium <6.0	Hydrolysis of casein	Lecithinase
anthracis	+	−	+	−	−	+	+	+	+	+	−	+	−	+	+	+
megaterium	+	−	−	−	−	ND	+	+	−	+	−	V	−	V	+	−
cereus	+	V	+	−	−	ND	+	+	+	+	−	+	+	+	+	+
thuringiensis	+	+	+	−	−	+	+	+	+	+	−	+	+	+	+	+
lichenformis	−	−	+	+	−	+	+	+	+	+	−	+	+	V	+	−
subtillis	−	−	−	+	−	+	+	+	+	+	−	+	+	V	+	−
pumilus	−	−	−	+	−	+	+	+	+	+	−	−	+	+	+	−
firmus	−	−	−	−	−	+	+	+	−	+	−	+	+	−	+	−
coagulans	V	−	+	+	−	−	−	+	+	+	−	V	+	+	V	−
polymyxa	−	−	+	−	−	−	−	+	+	+	+	+	+	V	+	−
macerans	−	−	+	+	−	−	−	+	−	+	+	+	+	−	−	−
circulans	−	−	V	+	−	V	V	+	−	+	−	V	+	V	V	−
stearothermophilus	V	−	−	+	+	V	−	V	−	+	−	V	−	+	V	−
alvei	V	−	+	−	−	V	−	+	+	+	−	−	−	+	+	−
laterosporus	−	+	+	+	−	V	−	+	−	+	−	+	−	−	+	+
brevis	−	−	−	+	−	−	−	+	−	+	−	V	−	−	+	−
sphaericus	V	−	−	−	−	V	V	+	−	−	−	−	−	−	V	−
larvae	−	−	+	−	−	−	+	−	−	+	−	V	−	−	+	ND
popilliae	−	+	+	−	−	−	+	−	−	+	−	−	−	−	−	ND
lentimorbus	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	ND
pasteurii	−	−	+	−	−	+	+	ND	−	ND	−	+	−	ND	V	−
pantothenticus	−	−	+	V	−	+	+	+	−	+	−	V	−	+	V	−

[a]+, greater than 85% of strains positive; −, greater than 85% of strains negative; V = variable character; ND, no data available.
From Reboli and Farrar (1988).

no gas bubbles form, repeat, using growth on chocolate agar. The test is not for cultures grown on blood agar because of the catalase present in the red blood cells. It can be done on egg-yolk agar.

Voges-Proskauer Test Broth (pH = 6.5)

Proteose peptone	7 g
Glucose	5 g
Sodium chloride	5 g
Distilled water	To 1 liter

J broth for V-P Reaction (pH = 7.3–7.5)

Tryptone	5 g
Yeast extract	15 g
Distilled water	To 1 liter

Add aseptically glucose 5 g/liter sterilized separately.

V-P Reaction (Acetyl methyl carbinol production)

Tubes of V-P broth should be inoculated in triplicate and tested for acetyl-methyl-carbinol production after incubation for up to 7 days. Mix 3 ml of 40% (w/v) sodium hydroxide with the culture and add 0.5 to 1 mg of creatine. Observe for the production of a red color after 30 to 60 min at room temperature. Fastidious insect pathogens should be grown on J-broth medium. For pH testing, before cultures incubated for 7 days are tested for acetyl-methyl carbinol, the pH is measured.

Anaerobic Agar (pH = 7.2)

Trypticase	20 g
Glucose	10 g
Sodium chloride	5 g
Agar	15 g
Sodium thioglycolate	2 g
Sodium formaldehyde sulfoxylate	1 g
Distilled water	To 1 liter

Inoculate a tube of anaerobic agar with a small loopful of nutrient broth culture by stabbing to the bottom of the culture tube. At incubation temperatures below 45°C the growth should be recorded at 3 and 7 days.

Growth in Sodium Chloride

Tubes of nutrient broth containing 0, 4, 7, and 10% (w/v) sodium chloride are inoculated and incubated. Observe for growth during up to 14 days of incubation.

Nitrate Reduction Broth (pH = 7.0)

Peptone	5 g
Meat extract	3 g
Potassium nitrate	1 g
Distilled water	To 1 liter

Reduction of nitrate to nitrite

Grow cultures in the above broth. After 3 and 7 days incubation, test by moistening a strip of potassium iodide/starch paper with a few drops of 1 N HC 1 and then touching the paper with a loopful of the culture. Observe for the production of a purple color indicating the presence of nitrite and for the accumulation of nitrogen gas. For a more rapid test, nitrate broth is inoculated and incubated at 35°C for 24 h. Sulfanilic acid and alpha-naphthol (0.25 ml each) are added. The presence of nitrite is indicated by an orange color that develops within 10 min.

Starch Hydrolysis Agar

Potato starch	1 g/10 ml cold distilled water
Nutrient agar	100 ml

Starch Hydrolysis Test

Inoculate duplicate plates of starch agar and incubate. At 3 and 5 days, flood one of the plates with 95% ethanol. After 15 to 30 min the unchanged starch will become white and opaque. Observe for a clear zone underneath and around the growth as an indicator of hydrolysis.

Casein Decomposition; Milk Agar

Agar	1 g in 50 ml of distilled water
Skim milk powder	5 g in 50 ml of distilled water

Casein Decomposition Test

Inoculate plates of milk agar with one streak of inoculum and examine after incubation at 7 and 14 days for clearing of the casein around and underneath the growth.

Citrate Utilization; Medium (pH = 6.8)

Trisodium citrate	1 g
Magnesium sulfate	1.2 g
Diammonium hydrogen phosphate	0.5 g
Potassium chloride	1 g
Agar	15 g
Distilled water	920 ml
Phenol red (0.04% w/v)	20 ml
Trace element solution*	40 ml

*$FeSO_4$, 200 mg; $ZnSO_4$, 10 mg; $MnCl_2$, 3 mg; H_3BO_3, 30 mg; $CoCl_2$, 20 mg; $CuCl_2$, 1 mg; $NiCl_2$, 2 mg; Na_2MoO_4, 3 mg; ethylene diamine tetraacetate, 500 mg; distilled water, 1 liter.

Citrate Utilization Reaction

Inoculate slants of citrate medium and incubate up to 14 days. Observe for production of a red (alkaline) color indicating utilization of organic acids.

Gelatin Liquefaction; Nutrient gelatin (pH = 7.0)

Use commercial nutrient gelatin medium or plain gelatin.

Gelatin	120 g
Distilled water	1 liter

As an alternative, nutrient agar supplemented with 0.4% gelatin may be used.

Gelatin Liquefaction Test

Inoculate tubes of nutrient gelatin and incubate at 28°C. Observe for liquefaction at 3 to 4 day intervals for 4 weeks. Before examination, hold the tubes at 20°C for about 4 h to allow unchanged gelatin to harden. If the gelatin does not harden at 20°C, liquefaction has occurred.

Lysozyme Resistance; Medium

Nutrient broth	99 ml
Lysozyme solution	1 ml

(10,000 enzyme units/ml of distilled water)

Lysozyme Test

Inoculate a loopful of a broth culture into a tube of the above medium and into a control tube of nutrient broth. After incubation for up to 7 to 14 days observe for growth or its absence. Members of the *B. cereus* group are resistant to lysozyme.

Tyrosine Decomposition; Agar

L-Tyrosine	0.5 g
Distilled water	10 ml
Sterile nutrient agar	100 ml

Tyrosine Test

Inoculate plates of the above agar with one streak of inoculum and incubate. Observe at up to 21 days for clearing of the tyrosine crystals around and below the growth. *B. cereus* and other members of the *B. cereus* group except for *B. anthracis* readily decompose tyrosine.

Phage typing using bacteriophage gamma has been used for typing *B. anthracis*, *B. thuringiensis*, *B. sphaericus*, and *B. stearothermophilus*.

Serologic tests (precipitation and complement fixation) have been used to separate *B. anthracis* from other *Bacillus* species, but antisera to *B. anthracis* react to some extent with other bacilli, limiting their usefulness. Agglutination tests have been found to be of little value, as these bacteria have the tendency to agglutinate spontaneously (Sievers and Zetterberg, 1940).

Bacillus anthracis and other *Bacillus* species can be differentiated by lectin agglutination assays (Cole et al., 1984). The agglutination can be carried out on a microscope slide. *B. anthracis* and *B. mycoides* give a positive agglutination with lectin from soybean (*Glycine max*). Agglutination with lectin from the snail *Helix pomatia* is positive only for *B. mycoides*. Spores have lectin receptors, and heating at 100°C decreases the agglutination.

A comparison of gas-liquid chromatography, DNA-DNA hybridization, API systems, and classic biochemical tests for speciation of some *Bacillus* species found API systems to be superior (O'Donnell et al., 1980). This impression was confirmed by Logan and Berkeley (1984) in their study of 600 *Bacillus* strains. API systems supplemented with spore examination, motility testing, and gas from carbohydrate fermentation were more rapid, accurate, and reproducible than classic biochemical tests. API and phage sensitivity testing are superior to other methods for distinguishing partially virulent and avirulent strains of *B. anthracis* from closely related species (Logan et al., 1985a).

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CHAPTER 1.2.19

Genera Related to the Genus *Bacillus*—*Sporolactobacillus*, *Sporosarcina*, *Planococcus*, *Filibacter* and *Caryophanon*

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Phylogenetic studies using comparisons of the oligonucleotide sequences of 16S rRNA have shown that taxa might group together which, according to classical taxonomy, would not have been found to be closely related. For instance, Fox et al. (1977) have shown that *Sporolactobacillus* may well be included in the genus *Bacillus*, and reference is not an exact matchPechman et al. (1976) and Fox et al. (1977) demonstrated that *Sporosarcina ureae* is more closely related to *Bacillus pasteurii* than are several other species of *Bacillus*. Similar studies suggest that a number of species or even genera, which, hitherto, have been placed in either nonsporeforming or Gram-negative taxa, should be placed close to the genus *Bacillus* and in particular to those species forming spherically shaped spores (Clausen et al., 1985; Stackebrandt et al., 1987). The dendrogram constructed according to these results shows a close neighboring relationship of *Filibacter limicola* to *Bacillus globisporus*, *Planococcus citreus* to "*B. aminovorans*," and *Caryophanon latum* to *B. sphaericus*. The *Bacillus* species forming spherical spores showed a higher degree of relationship to these nonsporeforming organisms than to each other and, in particular, higher than to the *Bacillus* species forming oval to ellipsoidal spores.

Also, the genus *Marinococcus,* comprising three species, has been separated from the genus *Planococcus*. Ventosa et al. (1990) described *Salinicoccus,* a genus phenotypically similar to *Marinococcus* and *Planococcus*. Both genera will be discussed within this chapter although their phylogenetical relationships to the family Bacillaceae and in particular to *Planococcus* are not known.

The genus *Bacillus* seems to be phylogenetically incoherent, and the spherical-sporeforming *Bacillus* species may represent a separate evolutionary line within the genus (Bonde, 1981; Logan and Berkeley, 1981; Priest, 1981). This is supported by rRNA analyses (Stackebrandt et al., 1987).

The phenotypic differentiation of the genus *Bacillus* from other genera mainly utilizes the following features: aerobic, sporeforming, rod-shaped, and Gram-positive cell wall. Although these four characteristics seem to be unequivocal, it is not always easy to provide evidence for them.

The ability to form spores is sometimes difficult to demonstrate for a number of strains or even species of the various sporeforming bacteria. Some organisms originally described as nonsporeformers were reclassified after the detection of spores, e.g., "*Lineola longa*" as "*B. macroides*" (reference is not an exact matchBennet and Canale-Parola, 1965) and "*Lactobacillus cereale*" as *B. coagulans* (Gordon et al., 1973), and there may be more of these. Many strains of recognized species are known to easily lose their ability to form spores after only a few transfers on culture media. Special treatment is necessary to avoid this loss which is, in most cases, not a loss of genetic information but loss of the potency of expressing a property. A general procedure for the induction of spore formation in a culture, especially for regaining of the ability to form spores, cannot be given. Various methods have been described, each having its own advantages with certain strains or species. Methods vary from supplementation of media with trace elements, especially manganese ions or soil extract, to variations in the conditions of incubation and supply of nutrients. Most of these methods or references to them are given in reviews Claus and Berkeley (1986) and Kalakoutskii and Dobritsa (1984).

The requirement that the genus *Bacillus* be Gram-positive remains, although strains or species that are Gram-variable or Gram-negative are accepted. In many species, Gram-positive reactions are seen only in very young cultures. Wiegel (1981) proposed that a differentiation should be made between Gram reaction, a staining phenomenon and Gram type, which refers to the structure of the cell wall as seen in thin sections in the electron microscope. And, indeed, in many cases it has been shown that the cell wall of a Gram-negatively staining strain is actually Gram-positive in structure.

This chapter was taken unchanged from the second edition.

Minimal Descriptions of the Bacillaceae

In the following, a minimal description of the two sporeforming and the three nonsporeforming genera placed—on the basis of phylogenetic studies—into the family of the Bacillaceae is given in comparison with the genus *Bacillus* as currently defined.

Genus *Bacillus*

Staining mostly Gram-positive, rods, peritrichously flagellated, endospores ellipsoidal or spherical, swelling or not swelling the sporangium, aerobic to facultatively anaerobic, mostly catalase positive.

Genus *Sporolactobacillus*

Gram-positive, rods, sparse-peritrichously flagellated, ellipsoidal endospores, swelling the sporangium, aerobic to microaerobic, catalase negative.

Genus *Sporosarcina*

Gram-positive, spheres occurring in pairs, tetrads, or packages, often only one flagellum per cell, spherical endospores, aerobic, catalase positive.

Genus *Planococcus*

Gram-positive, spheres occurring in pairs or tetrads, one to three flagella per cell, asporogenous, aerobic, catalase positive.

Genus *Filibacter*

Stains Gram-negative but its Gram type is positive, multicellular filaments, motile through gliding, asporogenous, aerobic, catalase positive.

Genus *Caryophanon*

Gram-positive, multicellular large rods, peritrichously flagellated, asporogenous, aerobic, catalase positive.

The Genus *Sporolactobacillus*

During the course of studies on the distribution of microorganisms in assorted chicken feed, Kitahara and Suzuki (1963) isolated an unusual strain of a lactic acid bacterium possessing certain characters intermediate between those of the genera *Bacillus* and *Lactobacillus*. The rod-shaped strain was Gram-positive, motile, formed endospores, was microaerophilic and catalase negative, and showed a typical homofermentative metabolism, producing D(−)-lactic acid. The authors created the new subgenus *Sporolactoba-*

cillus within the family Lactobacillaceae in order to accommodate this unusual bacterium. Later, *Sporolactobacillus* was transferred as an independent genus comprising only one species, *Sporolactobacillus inulinus*, into the family Bacillaceae (Kitahara and Toyota, 1972).

Similar strains were isolated by Nakayama and Yanoshi (1967a) and by Amemiya and Nakayama (1980). They were grouped with *S. inulinus* or were considered to belong to a new species for which the names "*S. laevas*," "*S. laevas* var. *intermedius,*" and "*S. racemicus*" have been proposed (Yanagida et al., 1987a). The genus was reviewed by Norris (1981), Norris et al. (1981), and Kandler and Weiss (1986).

Habitats

The original isolate, classified as *Sporolactobacillus inulinus*, was from chicken feed (Kitahara and Suzuki, 1963). Nearly all other strains of sporolactobacilli have been isolated from the rhizosphere or from soil around root hairs of a variety of wild plants collected in Japan and Southeast Asia (Nakayama and Yanoshi, 1967b; Amemiya and Nakayama, 1980). From a total of about 700 samples collected in the United States (foods, feed, soil, environment), Doores and Westhoff (1983) were able to isolate only two strains of *Sporolactobacillus*, both from soil samples. These authors concluded, therefore, that the incidence of sporolactobacilli in the environment is extremely low. Nakayama and Yanoshi (1967a) have pointed out that the rhizosphere represents a habitat where nutrients necessary for the growth of lactic acid bacteria may be provided by excretion from root hairs. Since the rhizosphere has a tendency to undergo drying and heating by sunlight impinging on the soil surface, it would favor, besides other microorganisms, the growth of motile, sporeforming, lactic acid bacteria.

Isolation

Many *Sporolactobacillus* strains have been isolated by Nakayama and Yanoshi (1967b) and by Amemiya and Nakayama (1980) using the following method:

Isolation Method 1 (Nakayama and Yanoshi, 1967a)

> The enrichment medium for *Sporolactobacillus* has the following composition (*Sporolactobacillus* broth):

Glucose	10.0 g
Polypeptone	10.0 g
Yeast Extract	10.0 g
Sodium citrate	0.027 g
KH_2PO_4	0.5 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.3 g
NaCl	0.01 g

MnSO$_4$ · 5H$_2$O	0.01 g
CuSO$_4$ · 5H$_2$O	0.001 g
CoCl$_2$ · 6H$_2$O	0.001 g
FeSO$_4$ · 7H$_2$O	0.001 g
Soil extract (see below)	100 ml
Distilled water	900 ml

Soil extract is prepared by autoclaving 100 g of garden soil mixed with 200 ml distilled water for 20 min at 130°C. The mixture is centrifuged to obtain a clear supernatant. The glucose broth is adjusted to pH 6.4 and is sterilized for 15 min at 115°C. Root material with root hairs and adherent soil from plants like *Trifolium repens*, *Allium japonicum*, *Ranunculus scelerata* and others is collected. Small pieces of the specimen are placed in a test tube containing a few ml of the sterile enrichment medium. In order to kill asporogenous organisms the tubes are heated at 80°C for 20 min and are incubated anaerobically at 30°C. In most tubes, clostridia will develop. On further incubation, however, the pH of cultures may drop below 4.0. Material from such cultures is streaked onto *Sporolactobacillus* agar composed of the broth described above supplemented with 10 g of calcium carbonate and solidified by 15 g of agar per liter of medium. After streaking; the agar surface is covered by a polyvinylidene chloride film, sterilized by autoclaving between filter papers, in order to depress the growth of aerobic bacteria. The plates are incubated aerobically at 30 to 37°C. Pin-point colonies with a transparent halo are picked, purified, and tested for the absence of catalase and the presence of endospores.

A selective technique for the enrichment and isolation of strains of the genus *Sporolactobacillus* has been developed by Doores and Westhoff (1983). Since the incidence of sporolactobacilli in the environment seems to be low, enrichment for these organisms is more likely to be successful than direct plating.

Isolation Method 2 (Doores and Westhoff, 1983)

Soil samples of about 5 g are collected aseptically in sterile 100-ml screw-cap bottles. To the soil sample, 50 ml of the following modified MRS broth is added:

α-Methylglucoside	10.0 g
Proteose peptone	10.0 g
Beef extract	5.0 g
Yeast extract	5.0 g
Tween 80	1.0 g
Ammonium citrate	2.0 g
Sodium acetate	5.0 g
Magnesium sulfate	0.1 g
Manganese sulfate	0.05 g
Disodium phosphate	2.0 g
Potassium sorbate	1.0 g
Bromocresol green	0.0224 g
Distilled water	1 liter

The medium is adjusted to pH 5.5 with 1 N acetic acid. After vigorous shaking for 2 min, the soil suspension is allowed to settle for about 5 min. Thereafter, the supernatant is transferred to a sterile 100-ml bottle, and the pH readjusted to 5.5 with 1 N or 0.1 N acetic acid or sodium hydroxide. The medium is incubated for 7 days at 30°C in a carbon dioxide incubator with a carbon dioxide level of 5% and a relative humidity of 98%. Thereafter, a 2-ml sample is transferred to a sterile test tube, which is then

heated for 5 min in a 80°C water bath to kill vegetative cells. Aliquots of 0.1 ml of the heat-treated sample are spread onto plates of modified MRS agar (modified MRS broth, pH 5.5 plus 15 g agar per liter of medium). The plates are incubated at 30°C. Colonies on the plates are screened for catalase production and benzidine reaction (both are negative for sporolactobacilli) as well as for the presence of spores and motility. The latter properties may be variable under the isolation conditions. Due to the specifity of the method, interference from environmental strains of *Bacillus* and *Lactobacillus* appears to be negligible.

Cultivation

Sporolactobacillus inulinus and other sporolactobacilli grow well on most media used for lactobacilli. In general, fermentable sugars are necessary for growth. Excellent growth has been observed on the GYP medium used by Kitahara and Suzuki (1963).

GYP Medium

Glucose	20.0 g
Yeast extract	5.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled water	1 liter

Incubation should be at 30 to 37°C under an atmosphere containing 5% carbon dioxide (Doores and Westhoff, 1983). Under air, only feeble growth may occur.

A series of commercially available media also support good growth under the conditions described. These include ATP agar (BBL) and lactobacilli MRS agar (Difco). For growing a large number of strains of the sporolactobacilli, Yanagida et al. (1987a) successfully used the following medium:

Sporolactobacillus Growth Medium

Glucose	20.0 g
Yeast extract	10.0 g
Peptone	10.0 g
Sodium acetate	10.0 g
MgSO$_4$> · 7H$_2$O	0.2 g
MnSO$_4$ · 4H$_2$O	0.01 g
FeSO$_4$ · 7H$_2$O	0.01 g
NaCl	0.01 g
Agar	20.0 g
Distilled water	1 liter

Adjust to pH 6.8. Incubate plates at 30°C.

Growth factor requirements have only been studied with a single strain which required biotin, pantothenic acid, leucine, and valine (Kitahara and Suzuki, 1963).

Maintenance and Preservation

Strains of *Sporolactobacillus* may be stored in liquid media for several months at −20°C if the acidity of cultures is neutralized after growth has ceased. Cultures can be freeze-dried by common procedures, for example, in 20% skim milk sup-

plemented with 5% *myo*-inositol. Maximum viability of sporulated cultures is not known.

Cultural and Microscopic Appearance

On agar media, sporolactobacilli form pinpoint colonies which are circular with convex shape, grayish white, and glistening. Due to the formation of copious amounts of lactic acid, colonies on media containing calcium carbonate are surrounded by clear haloes. Growth is feeble on agar slants. In agar shake cultures, uniform growth can be observed except for a 5-mm zone near the surface.

Sporolactobacillus strains are Gram-positive or Gram-variable. They form slender rods which are sometimes slightly bent. Cells measure 0.7–0.8 × 3–5 μm and occur singly, in pairs, and rarely in short chains. They are motile by a small number of long, peritrichously inserted flagella. Endospores are formed in certain media (see below).

When cultured in tomato-meat (TM) medium (see below) devoid of ammonium sulfate and calcium carbonate, a large proportion of vegetative cells form tadpole-like structures.

Endospore Formation

In most media the formation of endospores is very rare. A sporulation rate of 10^{-4} to less than 10^{-6} has been observed. However, a medium in which sporulation takes place at a higher rate has been developed by Kitahara and Lai (1967). In this medium the sporulation frequency can be increased up to 10% by incubating cultures under 5% CO_2 at 37°C.

TM Medium for Sporulation of *S. inulinus* (Kitahara and Lai, 1967)

Yeast extract	1.0 g
Meat extract	5.0 g
α-Methylglucoside	5.0 g
Ammonium sulfate	10.0 g
Tomato serum	200 ml
Distilled water	800 ml

Adjust the pH of the medium to 5.5 and add 20 g calcium carbonate (precipitated material). Doores and Westhoff (1981) found that the above medium without tomato serum was superior. Nakayama and Yanoshi (1967b) found that starch stimulated the formation of endospores.

Spores are ellipsoidal and 0.9 to 1.4 × 1.0–2.1 μm in size. They appear in the terminal to subterminal position. The sporangia are definitely swollen. As with other bacterial spore-formers, endospores contain dipicolinic acid.

Spores of only one *Sporolactobacillus* strain have been studied for heat resistance. They tolerate a treatment of 10 min at 70–80°C but not at 90°C. Decimal reduction times have been reported by Doores and Westhoff (1981).

Ultrastructure

Thin sections of vegetative cells show the usual cell wall type of Gram-positive bacteria (Kitahara and Toyota, 1972). The ultrastructure of spores is similar to those of *Bacillus* spores (Kitahara and Lai, 1967).

Chemotaxonomy

The cell wall of *Sporolactobacillus inulinus* contains *meso*-diaminopimelic acid, a large amount of polysaccharides, but no teichoic acid (Weiss et al., 1967; Okada et al., 1976). Uchida and Mogi (1973) examined the fatty acid composition of *Sporolactobacillus* strains and found odd-numbered, saturated anteiso- and iso-branched fatty acids. No unsaturated, even-numbered or cyclopropane fatty acids, which predominate in members of the genus *Lactobacillus*, have been detected.

Collins and Jones (1979) studied the isoprenoid quinone composition of strains of *S. inulinus*, "*S. laevas*," and "*S. racemicus*," as well as strains of some *Bacillus* species. In all strains, menaquinones with seven isoprene units (MK-7) predominate. In *Lactobacillus* strains, neither menaquinones nor ubiquinones were detected.

Genetic and Phylogenetic Relationships

Published values on the GC content of the DNA of *Sporolactobacillus inulinus* vary greatly. Using paper chromatography, Suzuki and Kitahara (1964) reported that the GC content of the type strain was 39.3 mol%. For the same strain, Kandler and Weiss (1986) found 38 mol% (T_m) and Miller et al. (1970) 47.3 mol%. This latter value was confirmed by Yanagida et al. (1987b) who found 46.8–47.0 mol% (T_m) for two strains of the species.

Miller et al. (1970) studied the DNA-DNA hybridization between strains of *S. inulinus*, *Lactobacillus plantarum*, and *Bacillus coagulans* and reported that there were no cross-reactions among these strains. Hybridization studies of Yanagida et al. (1987b), covering 33 *Sporolactobacillus* strains, have shown that the genus is genetically heterogenous, comprising five DNA homology groups (Table 1). Strain clusters found by a numerical taxonomic study of Yanagida et al. (1987a) did not correspond to the five DNA homology groups.

Results from cataloging the oligonucleotide pattern of ribosomal 16S RNA (Fox et al., 1977) have shown that *Sporolactobacillus* may well be included in the genus *Bacillus*. In later studies (Stackebrandt et al., 1987), it was found that *S. inulinus* was the only species possessing a phylo-

Table 1. Properties of the DNA homology groups of the *Sporolactobacillus* strains.

Homology group	GC content (mol%)	Intragroup homology	Species (number of strains)
1	46.8–50.2	88–100%	*S. inulinus* (2) "*S. laevas* var. *intermedius*" (1)
2	42.5–47.4	70–100%	"*S. laevas*" (12) "*S. laevas* var. *intermedius*" (3)
3	42.9–46.2	55–100%	"*S. racemicus*" (7) "*S. laevas* var. *intermedius*" (2)
4	42.5–45.8	79–100%	"*S. racemicus*" (2) "*S. laevas* var. *intermedius*" (2)
5	43.0	100%	"*S. laevas*" (2)

Adapted from Yanagida et al. (1987b).

genetic position differing from that of the other aerobic or facultatively anaerobic Gram-positive sporeformers examined up to now.

The close relationship of *Sporolactobacillus* and *Bacillus* can also be seen from chemotaxonomical studies (see above).

Physiological and Biochemical Properties

Although the genus is described as microaerophilic, all strains studied can be grown under air, even on agar plates. Strains tested showed improved growth, however, in the presence of 5% carbon dioxide. Some strains have been described as facultatively anaerobic (Nakayama and Yanoshi, 1967b).

Sporolactobacillus strains grow between 15–20 and 40°C with an optimum temperature at around 30°C. Although growth-limiting pH values are not known, cultures grow at a pH as low as 5.0. Since the pH of the medium decreases during growth in glucose broth to 3.8 to 3.2 (*S. inulinus*) or to pH 4.4 (other sporolactobacilli), growth may also be possible at a starting pH of 4.5.

Sporolactobacillus strains do not form catalase. Most strains need fermentable carbohydrates for growth. Acid without gas is produced by nearly all strains of the different DNA homology groups from glucose, fructose, mannose, maltose, sucrose, and trehalose. Acid formation from other carbohydrates is variable. In contrast to other strains studied, strains belonging to *S. inulinus* do not form acid from galactose (Yanagida et al., 1987a).

The type strain of *S. inulinus* has been shown to ferment hexoses exclusively to D(–)-lactic acid (homolactic acid fermentation) producing less than 1% of volatile acids or ethanol (Kitahara and Suzuki, 1963). According to Yanagida et al. (1987a), however, most strains of the genus Sporolactobacillus however, form both D(–) and DL-lactic acid. These discrepancies have to be clarified.

Identification

It is not yet possible to group *Sporolactobacillus* isolates according to their phenotypic properties with the type species of the genus, *S. inulinus*, or with any of the other sporolactobacilli described. Also, isolates cannot be grouped by phenotypic properties with the different DNA homology groups described by Yanagida et al. (1987b). Additional studies on phenotypic properties of the large number of available sporolactobacilli are now necessary. Because of the close relationship of *Sporolactobacillus* to the genus *Bacillus*, it is important to study the group by the same methods used for the characterization of *Bacillus* strains (Claus and Berkeley, 1986).

It has to be emphasized that a clear distinction between the genera *Sporolactobacillus* and *Bacillus* is also not possible. *Sporolactobacillus* strains, which all are characterized by the formation of lactic acid, can be separated from lactic acid-forming *Bacillus* species (*B. coagulans*, *B. smithii*, "*B. laevolacticus*," "*B. racemilacticus*") only by their temperature relationships and/or by their negative catalase reaction. Other phenotypic properties which may be used in the differentiation from these *Bacillus* species have not been studied.

While the lack of catalase in *Sporolactobacillus* has been considered as the main criterion for its separation from the genus *Bacillus*, it should be noted that certain *Bacillus* species also lack this enzyme. These include *B. azotoformans* (Pichinoty et al., 1983), *B. larvae*, *B. lentimorbus*, *B. popilliae*, certain strains of *B. stearothermophilus* (Gordon et al., 1973), and *B. pulvifaciens* (Nakamura, 1984).

Applications

Polysaccharide production of strains of the genus *Sporolactobacillus* has been studied by Amemiya and Nakayama (1980). Water-soluble fructans are produced from sucrose which show antitumor activity.

The Genus *Sporosarcina*

From urea-containing enrichment cultures, Beijerinck (1901) repeatedly isolated packet-forming coccoid bacteria. In contrast to all other sarcinae known at that time, the isolates were found to be motile by flagella and formed endospores. Beijerinck described these isolates as the new species "*Planosarcina ureae.*"

The species was later transferred by Löhnis (1911) to the genus *Sarcina*, which at that time comprised bacteria that were morphologically similar due to the formation of packets (sarcinae) of four or more cells. In physiological and biochemical properties, however, the various *Sarcina* species of Löhnis differed from each other substantially. Therefore, "*Sarcina ureae*" was later separated from the genus *Sarcina* and transferred to the genus *Sporosarcina* (Kocur and Martinec, 1963; MacDonald and MacDonald, 1962) as previously proposed by Orla-Jensen (1909) and by Kluyver and van Niel (1936).

Strains of two species of the genus *Sporosarcina* are available now in pure culture and have been extensively described: *S. ureae* (Beijerinck, 1901) Kluyver and van Niel, 1936[AL], and *S. halophila* Claus, Fahmy, Rolf and Tosunoglu, 1984[VP]. Beijerinck (1901) briefly mentioned another sporeforming sarcina which he named "*Urosarcina dimorpha*," which developed only on media containing horse urine gelatin, but it is not certain whether pure cultures were isolated. Attempts to reisolate "*U. dimorpha*" have been unsuccessfull (D. Claus, unpublished observation). The only other described taxon of the genus is "*Sarcina pulmonum*," a motile, sporeforming urea-degrading organism isolated from clinical material, but these strains were subsequently lost. According to Gibson (1935), these isolates were probably identical with *Sporosarcina ureae*.

S. ureae and *S. halophila* are considered to be nonpathogenic for humans, animals, and plants. The genus has been reviewed by Claus (1981), Norris (1981), and Claus and Fahmy (1986).

Habitats

Like other sporeforming bacteria, *Sporosarcina ureae* is widely distributed in soil. Fertile soils may contain up to 10^4 sarcinae/g (Gibson, 1935; Pregerson, 1973). The bacterium probably has also been isolated from liquid manure (Sames, 1898). A single isolate has also been reported from sea water (Wood, 1946). Pregerson (1973) has found *S. ureae* to be widely distributed in the United States and in various other parts of the world. According to her studies, the primary habitat of the organism appeared to be concentrated in certain urban soils closely associated with the activities of humans and, especially, dogs.

The prevalence of the bacterium in fertile soils and certain other urea-containing places, its resistance against the inhibitory effect of up to 5–10% urea, and its ability to produce urease suggest that *S. ureae* plays an active part in the decomposition of urea in natural habitats.

The sporeforming "*Sarcina pulmonum*" was isolated at the beginning of this century by several workers from sputum and from the respiratory tract in cases of phthisis. Although for some time it was considered to be responsible for severe infections of the lungs, its pathogenicity has been questioned (Lehmann and Neumann, 1927; Gibson, 1935). Recently, a bacterial isolate from a bronchial biopsy in a young child with cystic fibrosis has been identified as *Sporosarcina ureae*. It was considered, however, not to be directly related to the disease (Chomarat et al., 1990).

S. halophila has been isolated from salt marsh soils (Claus et al., 1983), saline soils, and ponds of solar salterns (Ventosa et al., 1983). Its moderately halophilic characteristics, together with its obligate requirements for sodium, magnesium, and chloride ions, as well as its optimum temperature for growth (about 30°C), suggest that salt marsh and saline soils are the natural habitat of this species.

Isolation

Selective methods for the enrichment of the two accepted *Sporosarcina* species in liquid media have not been reported. The selective enrichment method used by Beijerinck (1901) for the isolation of *S. ureae* does not seem to work well. Strains of both species, however, can be isolated by plating soil dilutions on appropriate agar media.

ISOLATION OF *SPOROSARCINA UREAE*   The presence of *S. ureae* in many soil samples has been demonstrated by a simple and effective method (Gibson, 1935). Since 10% urea, as used by Gibson, may inhibit the growth of *S. ureae* from many soils, urea should be used at a concentration of only 3–5%. At this concentration, the growth of a high percentage of the bacterial soil flora is strongly inhibited. This is specifically true for *Bacillus mycoides*, which will readily overgrow most soil dilution plates at urea concentrations below 3%.

Method 1 for the Isolation of *S. ureae* (Gibson, 1935; Modified by Claus, 1981)

> To 1 liter of nutrient agar, add 30, 50, or 100 g of urea. Sterilize the media at 121°C for 15 min and pour into petri dishes. Suspend about 5 g of a soil sample (fertile soil, air-dried) in 20 ml of sterile water and prepare soil dilutions (10^{-1} and 10^{-2}). Plate 0.1 ml of the soil suspension and of the two dilutions on the agar and incubate at about 25°C. The soil suspension may be heated, but there is little or

no advantage to doing so because the majority of the organisms that develop on the media are sporeforming bacteria.

After 3–5 days, examine only cream or pale yellow to bright orange colored colonies under low magnification and transmitted light. Colonies of *S. ureae* can be recognized as round and black at a magnification of about 10×, and by their coarsely granulated structure, especially at the edges of the colonies, at a magnification of about 50×. With some experience, colonies of *S. ureae* may be selected with rather high certainty. Similar types of colonies, however are often formed by strains of *Bacillus megaterium*. Therefore, it is advisable to compare the colony types on isolation plates with those of known strains of the two species.

Prepare slides from selected colonies for observing the typical morphology of cells and confirm the provisional identification of isolated strains of *S. ureae* testing the motility and the production of spores (see below, under maintenance). Pure cultures are obtained by restreaking suspensions prepared from single colonies onto nutrient agar containing 2% urea.

Pregerson has isolated about 50 strains of *S. ureae* from 198 different soil samples and has proposed the following method for isolating the organism. A good source for isolation is soil from the base of trees where dogs have urinated.

Method 2 for the Isolation of *S. ureae* (Pregerson, 1973)

Tryptic soy-yeast (TSY) agar containing (per liter of distilled water); 27.5 g Difco tryptic soy broth, 5.0 g Difco yeast extract, 5.0 g glucose, and 15.0 g Difco agar, is adjusted with 1 N NaOH to pH 8.5 before autoclaving. Add aseptically filter-sterilized urea to give a final concentration of 1% (w/v) and pour the medium into petri dishes. Suspend 1 g of a soil sample in 15 ml of distilled water and mix the slurry with a Vortex mixer. Spread 0.1 ml of a series of dilutions on plates using a sterile bent glass rod. Prepare triplicate plates at 10^{-1} and 10^{-2} and duplicate plates at 10^{-3} and 10^{-4} dilutions.

Incubate at 22°C and examine the plates on the third day and then daily with a dissecting microscope. Colonies of *S. ureae* show a uniform-surface granularity, smoothly opaque interiors, and an orange or cream color. Prepare slides from selected colonies for observing cell morphology and motility and the production of spores (see below under maintenance), and isolate pure cultures.

The isolation of *S. ureae* from a certain soil sample often is not reproducible due to the irregular background growth developing on dilutions plates. A more selective method suppressing such growth or a selective liquid enrichment method will allow the isolation of this species also from soils where it is only present in low numbers.

Isolation of *Sporosarcina halophila* (Claus et al., 1983)

Two different agar media have been used for isolation. Bacto Marine Agar 2216 (Difco) is composed of:

Bacto peptone	5.0 g
Bacto yeast extract	1.0 g
Ferric citrate	0.1 g
Sodium chloride	19.45 g
Magnesium chloride	8.8 g
Sodium sulfate	3.24 g
Calcium chloride	1.8 g
Potassium chloride	0.55 g
Sodium bicarbonate	0.16 g
Potassium bromide	0.08 g
Strontium chloride	0.034 g
Boric acid	0.022 g
Sodium silicate	0.004 g
Sodium fluoride	0.0024 g
Ammonium nitrate	0.0016 g
Disodium phosphate	0.008 g
Agar	15.0 g
Distilled water	1 liter

Adjust the pH to about 7.5. The medium is sterilized at 121°C for 15 min and poured into petri dishes.

Alternatively, the following medium may be used:

Peptone	5.0 g
Meat extract	3.0 g
Sodium chloride	30.0 g
Magnesium chloride	5.0 g
Agar	15.0 g
Distilled water	1 liter

Adjust the pH to about 7.5

Suspend about 5 g of a sample of salt marsh soil in 20 ml of sterile tap water. Heat the suspension at 70°C for 10 min. Alternatively, the soil suspension may be treated with ethanol, final concentration 50% (v/v), for 1 h (to kill vegetative bacterial cells). Prepare soil dilutions in sterile water (10^{-1} to 10^{-4}). Plate 0.1 ml of the dilutions on agar medium.

After 3 days of incubation at 30°C, mainly pigmented (yellow, orange, or pink) colonies of various sizes of *Bacillus* species will have developed. Inspect the more crowded plates at a magnification of about 50× for round pin-point colonies which show, in transmitted light, a coarsely granulated structure at least at the edges of the colonies. The same colonies appear black under a magnification of only 10×. Check material of such colonies by phase contrast microscopy for the presence of sarcinae, which usually are motile or may have formed endospores.

For purification, suspend cell material taken from appropriate colonies in a drop of nutrient broth and streak onto plates of one of the agar preparations mentioned above. Colonies of *S. halophila* form an orange pigment which normally is not seen with the pin-point colonies developing on the isolation plates.

Fahmy et al. (1985) have observed that spores of *S. halophila* formed in pure culture will germinate only at a very low frequency in nutrient broth supplemented with 3% sodium chloride and 0.5% magnesium chloride, although growth of vegetative cells in such media is excellent. In contrast, a high percentage of spores readily germinate in nutrient broth supplemented with double-concentrated sea water (see below). This observation may be helpful in developing new media and methods for the selective enrichment and isolation of additional strains of *S. halophila* and to enumerate the species in natural samples.

Cultivation

Sporosarcina ureae grows well in normal nutrient broth. A more suitable medium contains, in addition, 1% urea or 0.5% ammonium chloride. In the latter case, the pH of the medium should be adjusted to 8.0 or 8.5. Since the organism is strictly aerobic, shaking is recommended to obtain high cell densities. The optimum temperature for growth is about 25°C.

A defined medium for *S. ureae* has been described by Goldman and Wilson (1977), which yields 5–6 g dry weight of bacteria per liter of culture.

S. ureae Growth Medium

L-Asparagine · H$_2$O, or L-Glutamine	30.0 g
KCl	3.4 g
K$_2$HPO$_4$	0.25 g
(NH$_4$)$_2$SO$_4$	0.2 g
MgSO$_4$ · 7H$_2$O	0.05 g
FeSO$_4$ · 7H$_2$O	0.0025 g
MnCl$_2$ · 4H$_2$O	0.00025 g
D-Biotin	0.001 g
L-Cysteine	0.005 g
Distilled water	1 liter

Prior to sterilization, the medium is adjusted to pH 8.7 with 1 M NaOH and supplemented with NaCl to a final concentration of 0.05 M Na+. Sterilized solutions of biotin (0.4 mg/ml), L-cysteine (2 mg/ml), and (NH$_4$)$_2$SO$_4$ (80 mg/ml) were added to the separately sterilized medium.

Pregerson (1973) has pointed out that the growth factor requirements of strains of *S. ureae* may vary substantially. From 61 isolates studied, 13 grew on a mineral acetate medium without added growth factors, but 37 strains required biotin either singly or in combination with niacin and/or thiamine. Some of these strains also needed aspartate. Eleven strains showed more complex growth factor requirements. Most of the strains could utilize acetate, butyrate, or glutamate as a sole source of carbon and energy. Glutamate, however, generally could not be used by *S. ureae* as the sole nitrogen source.

According to the definition of Larsen (1962), *S. halophila* is a slightly halophilic bacterium. It is one of the few "marine" bacteria which shows an obligate growth requirement for sodium, magnesium, and chloride ions. Sodium cannot be replaced by potassium nor chloride by sulfate. The salt requirements of the strains apparently reflect the natural habitat of the species.

In the presence of 30 to 50 g NaCl (maximum 150 g) and 5 g MgCl$_2$ per liter, all strains of the species studied show profuse growth on a variety of complex media. The pH optimum for growth is between pH 7.0 and 9.0 with an optimum at about pH 7.8. Strains grow from 15 to 35 or 40°C. The optimum temperature of growth is around 30°C. The species is strictly aerobic. It grows well on the following medium:

S. halophila Growth Medium

Peptone from meat	5.0 g
Meat extract	3.0 g
NaCl	30.0 g
MgCl$_2$	5.0 g
Agar	15.0 g
Distilled water	1 liter

Adjust the pH to 7.8.

Good growth is also observed on Bacto Marine Broth 2216 or Bacto Marine Agar 2216 (Difco). A chemically defined medium for *S. halophila* has not yet been developed.

Maintenance and Preservation

Vegetative cultures of *S. ureae* and of *S. halophila* may be kept for more than 8–12 weeks at about 4°C on nutrient agar slants if protected from drying. In tightly closed tubes, sporulated cultures (see below) may survive for several years if stored at 4°C. Sporulated cultures (see below) remain viable for more than one year.

For long-term maintenance, vegetative cells and spores of *S. ureae* and *S. halophila* can be preserved for years without significant loss in viability by lyophilization in skim milk (20% w/v) in the presence of 5% *myo*-inositol. Vegetative and sporulated cells can also be preserved for long periods in liquid nitrogen in the presence of a suitable cryoprotective agent like 5% (v/v) dimethylsulfoxide or 10% (v/v) glycerol (D. Claus, unpublished observations).

Cultural and Microscopic Appearance

Colonies of *Sporosarcina ureae* on agar are gray, opaque, circular, and slightly convex with an entire margin. On some media, a yellowish, brownish, or orange nondiffusible pigment may be produced. *S. halophila* forms round, smooth, and opaque colonies. Irrespective of the medium used, an orange nondiffusible pigment is formed.

The typical cell shape of both species is spherical. Often cells are modified by interfacial flattening in the cell aggregates. Especially in *S. halophila*, oval- to egg- or pear-shaped cells are also formed. The diameter of spherical cells is 1.0–2.5 μm. Oval cells of *S. halophila* measure 1–2 × 2–3 μm. Division walls usually are at right angles to one another.

In cultures of both species, various cell aggregate patterns can be found. Single cells, pairs, threes, tetrads, or packets are common or may predominate, depending on the medium used. In older cultures, aberrant forms often can be found: abnormally large cells appearing singly or within packets, short chains, or irregular packets. Apparently, this is due to an asynchronous cell division.

Cells and aggregates usually show a tumbling motility. With *S. ureae*, this is best observed using material from nutrient broth containing 1% urea (Kocur and Martinec, 1963). Most often, a single flagellum is formed per cell. It is difficult, however, to determine the precise number and location of flagella in cell packets (Sersen et al., 1983). *S. halophila* forms motile cells in young cultures grown on the agar media decribed above.

Endospore Formation

Both species form endospores under certain growth conditions. They are highly refractile, round, 0.5–1.5 μm in diameter, and located centrally or laterally. Like those of most *Bacillus* species, they resist heating for 10 min at up to 80°C or even more.

Spores of *S. ureae* can be obtained on one of the following sporulation media if the incubation temperature is lower than 22°C.

Sporulation Medium 1 for *S. ureae* (Gibson, 1935)

Peptone	5.0 g
Meat extract	5.0 g
Ammonium chloride	5.0 g
Agar	15.0 g
Distilled water	1 liter

Adjust the pH to 6.8–7.0.

Sporulation Medium 2 for *S. ureae* (Claus, 1981)

Peptone	5.0 g
Meat extract	3.0 g
Manganese chloride	50.0 mg
Agar	20.0 g
Distilled water	1 liter

The pH is not adjusted. After sterilization (20 min at 121°C) 20 ml of a filter-sterilized solution containing 10% (w/v) urea is added per liter of medium.

Sporulation Medium 3 for *S. ureae* (MacDonald and MacDonald, 1962)

Yeast extract	2.0 g
Peptone	3.0 g
Glucose	4.0 g
Malt extract	3.0 g
K_2HPO_4	1.0 g
$(NH_4)_2SO_4$	4.0 g
$MgSO_4$	0.8 g
$CaCl_2$	0.1 g
$MnSO_4 \cdot H_2O$	0.1 g
$FeSO_4 \cdot 7H_2O$	0.001 g
$ZnSO_4$	0.01 g
$CuSO_4 \cdot 5H_2O$	0.01 g
Agar	30.0 g
Water	1 liter

Adjust the pH before sterilization to 8.8–9.0.

At 22°C, all strains isolated by Pregerson (1973) showed production of spores on the latter medium. However, considerable variation in the onset and extent of sporulation was observed.

Some strains produced only a few spores after 4 weeks of growth, while others produced an abundance of spores within 4 days. Some strains produced spores more readily when the peptone of the medium was replaced by casamino acids (Difco). Crowded conditions on plates apparently stimulated spore formation.

Spore formation of *S. halophila* initially was good on Difco Marine Agar 2216 (composition see above). This property was totally lost by all strains after purification and few transfers on this medium. However, with most strains, sporulation could be retained by growing cultures on the seawater agar of Lyman and Fleming (1940) and by incubating the cultures at 22°C (Fahmy et al., 1985).

Seawater Agar

Bacto peptone (Difco)	5.0 g
Yeast extract	1.0 g
Ferrous phosphate · 4H₂O	0.1 g
Manganese chloride	0.01 g
Agar	15.0 g
Synthetic seawater (see below)	1 liter

The pH of the medium is adjusted to 7.1 before sterilization.

Synthetic Seawater

Distilled water	1 liter
Sodium chloride	24.32 g
Magnesium chloride · 6H₂O	10.99 g
Sodium sulfate	4.06 g
Calcium chloride · 2H₂O	1.51 g
Potassium chloride	0.69 g
Sodium bicarbonate	0.20 g
Potassium bromide	0.10 g
Strontium chloride · 6H₂O	0.042 g
Boric acid	0.027 g
Sodium silicate · 9H₂O	0.005 g
Sodium fluoride	0.003 g
Ammonium nitrate	0.002 g
Ferrous phosphate · 4H₂O	0.001 g

Like endospores of the genus *Bacillus*, the endospores of the two *Sporosarcina* species contain the spore-specific compound dipicolinic acid, which has never been found in vegetative bacterial cells (Fahmy et al., 1985; MacDonald and MacDonald, 1962; Thompson and Leadbetter, 1963).

Ultrastructure

Studies on the fine structure of vegetative cells, including regular surface layers, have been published for *S. ureae* (Beveridge, 1979; Beveridge, 1980; Engelhardt et al., 1986; Mazanec et al., 1965; Robinson and Spotts, 1983; Silva et al., 1973; Stewart and Beveridge, 1980). The ultrastructure of spores of both *Sporosarcina* species is similar to that of *Bacillus* spores (Silva et al., 1973; Robinson and Spotts, 1983; Fahmy et al., 1985).

Chemotaxonomy

Whereas the cortex of *Sporosarcina* spores, like all *Bacillus* species studied, contains *meso*-diaminopimelic acid, this compound has not been detected in the walls of vegetative cells of both *Sporosarcina* species (Claus et al., 1983; Ranftl, 1972; Schleifer and Kandler, 1972). The peptidoglycan types found in vegetative cells, however, differ with the two species. For *S. ureae*, the lys-gly-D-glu type has been described (Linnett et al., 1974; Schleifer and Kandler, 1972), whereas in *S. halophila*, the orn-D-asp type is found (Kandler et al., 1983). Also in cell wall preparations of *S. halophila*, high amounts of a gamma-D-glutamyl polymer have been detected, but this compound is not present in *S. ureae* (Kandler et al., 1983).

The menaquinone system of both species is MK-7. This corresponds to the one found in most species of the genus *Bacillus* (Claus et al., 1983; Collins and Jones, 1981; Yamada et al., 1976).

Except for the observation that cell membranes of *S. ureae* contain phosphatidylethanolamine (Komura et al., 1975), other data of chemotaxonomic relevance have not been published.

Genetic and Phylogenetic Relationship

The GC content for 11 strains of *S. ureae* has been found to be in the range of 40.0–41.5 mol% (T_m) (Bohácek et al., 1968b) and for three other strains, from 40.6–40.8 mol% (T_m) (Claus et al., 1983). DNA-DNA hybridization studies have been performed only with two strains of the species. The binding between these is 93% (Fahmy, unpublished observations).

The GC content of 22 strains of *S. halophila* was determined to be in about the same range (40.1–40.9 mol%; T_m). According to DNA-DNA hybridization studies, the species is homogenous; the homology of the DNA of the 22 strains studied is from 72–100%. The DNA homology between the type strains of the two species, however, is only 36% (Claus et al., 1983).

A close relationship of the sporeforming genera *Sporosarcina* and *Bacillus* was already suggested by Beijerinck (1901), who observed the occurrence of soil bacteria morphologically intermediate between *S. ureae* and *Bacillus megaterium*.

The genetic relationship of the two genera was first studied by Herndon and Bott (1969) using DNA-RNA hybridization methods. By comparative 16S rRNA cataloging, reference is not an exact matchPechman et al. (1976) and Fox et al. (1977) have shown that *S. ureae* is more closely related to *Bacillus pasteurii* than are several other species of *Bacillus*. These authors concluded that *S. ureae* should best be classified as a member of the genus *Bacillus*. In the eighth edition of *Bergey's Manual of Determinative Bacte-riology*, however, the genus *Sporosarcina* was given generic rank in the family Bacillaceae (Gibson, 1974).

The close phylogenetic relationship of *S. ureae* to members of the genus *Bacillus* has been confirmed by Stackebrandt et al. (1987). *S. ureae* forms spherical endospores and is found to cluster with other *Bacillus* species forming spherical spores (e.g., *B. pasteurii*, *B. sphaericus*). This group, which also includes some non-sporeforming bacteria, is separated from those *Bacillus* species forming ellipsoidal endospores (e.g., *B. subtilis*, *B. cereus*, *B. megaterium*, *B. pumilus*). These and other studies on the bacterial phylogeny suffer from the fact that very often the type strain of the species were not used.

The phylogenetic relationship of *S. halophila* to *S. ureae* or to members of the genus *Bacillus* has not been reported.

The nucleotide sequence of the 5S rRNA of *S. ureae* has been published by Park et al. (1988).

Physiological and Biochemical Properties

The general properties of *S. ureae* have been described by Kocur and Martinec (1963) and by MacDonald and MacDonald (1962). The results have been confirmed using the methods described by Gordon et al. (1973) for the characterization of *Bacillus* species (D. Claus, unpublished observations). Properties of *S. halophila* have been studied by Claus et al. (1983), using, as far as possible, the same methods. All media, however, were supplemented with 3% NaCl and 0.5% $MgCl_2$. The properties of the two species are listed in Tables 2 and 3.

Identification

The two *Sporosarcina* species can easily be differentiated by the series of phenotypic properties listed in Table 2. They can clearly be

Table 2. Differentiating physiological and biochemical properties of the two *Sporosarcina* species.

Character	*S. ureae*	*S. halophila*
Growth		
In nutrient broth	+	−
In nutrient broth plus 10% NaCl + 0.5% $MgCl_2$	−	+
Hydrolysis of:		
Starch	−	+
Pullulan	−	+
Casein	−	+
Gelatin	−	+
Nitrite from nitrate	+	−
Urease	+	−
Tyrosine decomposed	+	−

Symbols: +, positive; −, negative.
Adapted from Claus et al. (1983).

Table 3. Other properties of the two *Sporosarcina* species.

Character	*S. ureae*	*S. halophila*
Growth		
In nutrient broth	+	–
In nutrient broth plus 15% NaCl + 0.5% MgCl₂	–	+
At 30°C	+	+
37°C	V	+
40°C	–	V
45°C	–	–
At pH 5.7	–	–
Anaerobically	–	–
Catalase	+	+
Oxidase	+	+
Arginine dihydrolase	V	–
Phenylalanine deaminase	V	–
Phosphatase	–	V
Lecithinase	–	–
Indole	–	–
Voges-Proskauer test	–	–
Citrate utilized	–	–
Hydrolysis of		
Dextran	–	–
DNA	V (weak)	+
Chitin	–	–
Cellulose	–	–
Tween 80	V	–
Gas from nitrate	–	–

Symbols: +, positive; –, negative; V, variable.
Adapted from Claus et al. (1983).

separated from morphologically similar cocci by the characters listed in Table 4.

Other Studies

Beijerinck (1901) and Gibson (1935) indicated that urea can be hydrolyzed by *Sporosarcina ureae* at concentrations of up to 5–10%. Urease was characterized as an exoenzyme by Pel'ttser (1969) and quantified by Kaltwasser et al. (1972). The presence of enzymes required for a functioning urea (ornithine) cycle in *S. ureae* has been demonstrated by Gruninger and Goldman (1988). A strain of *S. ureae* was included in studies on the esterase pattern of psychrotrophic *Bacillus* species (Higashi and Johnson, 1986).

A bacteriophage of *S. ureae* was first isolated by Kluckhohn and Spotts (1986). Jensen and Stenmark (1970) characterized the control mechanism of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthetase and have shown that the control pattern resembled that of species in the genus *Bacillus*.

Applications

Intracellular L-phenylalanine dehydrogenase of *Sporosarcina ureae* can be used for the efficient enzymatic transformation of phenylpyruvate to L-phenylalanine (Asano and Nakazawa, 1985; Campagna and Bückmann, 1987). The enzyme was highly purified and crystallized by Asano and Nakazawa (1985). It can also be used for the high yield synthesis of other L-amino acids from their keto analogs by combination with formate dehydrogenase (Asano and Nakazawa, 1987).

The Genus *Planococcus*

Motile cocci were first isolated by Ali-Cohen (1889) and were named "*Micrococcus agilis*." Migula (1894) established, for motile cocci, the new genus "*Planococcus*," which he placed into the family Coccaceae (Migula, 1900). However, most bacterial taxonomists continued to include motile cocci in the genus *Micrococcus*, as they are very similar in cultural and biochemical characteristics to true micrococci. Bohácek et al. (1967, 1968a) determined the GC content of some micrococci and concluded that non-motile cocci are not taxonomically related to motile strains. Kocur et al. (1970) studied the taxonomy of motile cocci and revived and amended the genus *Planococcus* Migula 1894 with the type species, *P. citreus*.

Only two species are now recognized in the genus, *P. citreus* Migula 1894, 236[AL] and *P. kocurii* Hao and Komagata 1986[VP]. As a result of chemotaxonomical studies, the species *P. halophilus* reference is not an exact matchNovitski and Kushner 1976, 53[AL] was transferred to a new genus called *Marinococcus* (Hao et al., 1984).

Habitats

Planococci have been isolated from marine environments, sea water (ZoBell and Upham, 1944), marine clams (Leifson, 1964), fish-brining tanks (Georgala, 1957), and boiled and frozen shrimps and prawns (Alvarez, 1982; Hao and Komagata, 1985). They play a role in food hygiene because they may cause putrefaction of shrimps during storage (Alvarez, 1982).

Isolation

No selective medium for the isolation of planococci has been devised. For their isolation, seawater agar or nutrient agar supplemented with 10% NaCl may be used.

Seawater Agar

Beef extract	10 g
Peptone	10 g
Tap water	250 ml
Agar	20 g
Seawater	720 ml

Adjust to pH 7.20

Table 4. Differentiating properties of the moderately halophilic cocci.

Character	Planococcus citreus[a]	Planococcus kocurii[b]	Marinococcus halophilus[c]	Marinococcus albus[c]	Marinococcus hispanicus[d]	Salinicoccus roseus[e]	Sporosarcina ureae[f]	Sporosarcina halophila[f]
Motility	+	+	+	+	−	−	+	+
Cell arrangement:								
Irregular clusters	−	−	−	−	−		−	−
Tetrads	+	+	+	+	+		+	+
Endospores	−	−	−	−	−	−	+	+
Pigment	YO	YO	YO	CW	RO	PR	D	YO
Growth in presence of:								
0% NaCl	+	+	−	−	−	−	+	−
15% NaCl	+	−	+	+	+	+	−	+
Oxidase	−	−	−	+	+	+	+	+
Urease	−	−	−	+	D	−	+	−
Hydrolysis of:								
Gelatin	+	+	+	−	+	+	−	+
DNA	+	+	−	+	D	+	+	D
Nitrate to nitrite	−	−	−	+	D	+	+	+
Acid from:								
Glucose	+	D	+	−	D	−	−	−
Maltose	−	−	+	−	D	−	−	−
Sucrose	−	−	+	−	−	−	−	−
GC content (mol%)	48–52	39–41	46.4	44.9	45.6–49.3	51.2	40.0–41.5	40.1–41.9
Menaquinone system	MK-7, MK-8	MK-7, MK-8	MK-7	MK-7	MK-7, MK-8	MK-6[g]	MK-7	MK-7
Cell wall type	L-Lys-D-glu	L-Lys-D-glu	meso-A$_2$pm	meso-A$_2$pm	meso-A$_2$pm	L-Lys-gly$_5$	L-Lys-gly-D-glu	Orn-D-asp

[a]Kocur, 1986.
[b]Hao and Komagata, 1985.
[c]Hao et al., 1984.
[d]Marquez et al., 1990.
[e]Ventosa et al., 1990.
[f]Claus and Fahmy, 1986.
[g]B. Tindall, personal communication.
Symbols: +, positive; −, negative; D, differs among strains; pigment; YO, yellow-orange; CW, creamy white; RO, reddish orange; PR, pink-red.

Cultivation

Planococci can be cultivated on seawater agar, on peptone-yeast agar, or on nutrient agar supplemented with up to 10% sodium chloride.

Maintenance and Preservation

Cultures of planococci may be maintained on slants of seawater agar at 4°C for about 6 months. Strains can be preserved in a freeze-dried state or in liquid nitrogen for long periods.

Cultural and Microscopic Appearance

Colonies of planococci are circular, slightly convex, smooth, and yellow-orange in color due to water-insoluble carotenoid pigments. Pigmentation as well as the type of carotenoids produced may be influenced by the concentration of sodium chloride in the medium and by the age of the culture (Thirkell and Summerfield, 1980). A hydrostatic pressure of 40 MPa has no influence on the pigment production of *P. citreus* (Courington and Goodwin, 1955).

The genus includes Gram-positive, nonsporforming, spherical cells of 1.0 to 1.2 μm in diameter, occurring singly, or in pairs, tetrads, or clumps. Motile cells usually have one to three flagella. Endospores are not formed.

Ultrastructure

The fine structure of the cells of *Planococcus citreus* is similar to that of other Gram-positive, catalase-positive cocci. The cell wall is double layered. Its thickness varies with the cell age from 25–35 nm (Kocur, 1986).

The concentration of salt in the medium affects the amount of membrane in the cell of *P. citreus*. Salt concentrations above and below the normal 3% of sea water apparently reduce the amount of membrane material present (Thirkell and Summerfield, 1977a).

Chemotaxonomy

The cell wall peptidoglycan of both *Planococcus* species is of the L-lys-D-glu type (Schleifer and Kandler, 1970; Hao and Komagata, 1985). No teichoic acid was found in the cell wall of planococci (Endresen and Oeding, 1973). *Marinococcus* species, in contrast, have a cell wall of the *meso*-diaminopimelic acid type.

In common with other Gram-positive bacteria, *P. citreus* contains *anteiso* $C_{15:0}$ as the major component of free fatty acids. Phospholipids detected in planococci are similar to that of *Sporosarcina* and include phosphatidylethanola-

mine, cardiolipin, and phosphatidylglycerol (Yamada et al., 1976; Thirkell and Summerfield, 1977b; Hao and Komagata, 1985).

Planococci contain normal menaquinones, with MK-8 and MK-7 in about equal amounts. A small amount of MK-6 is also present (Hao and Komagata, 1985).

Genetic and Phylogenetic Relations

The analysis of the GC content revealed two groups among *Planococcus* species. Strains of *P. citreus* show 47 to 52 mol% (T_m), while the DNA base composition of *P. kocurii* is in the range of 39–42 mol% (T_m) (Bohácek et al., 1967, 1968; Hao and Komagata, 1985). Strains of *Marinococcus*, earlier grouped with *Planococcus*, have a GC content in the range of 43.9 to 46.6 mol% (T_m) (Hao et al., 1984). DNA-DNA hybridization studies have not been performed within the genus.

A comparative analysis of 16S rRNA sequences has shown that planococci bear no specific relationship to the genera *Micrococcus* and *Staphylococcus* (Stackebrandt and Woese, 1979). Planococci show a specific relationship to the genus *Bacillus*, particularly to *Bacillus pasteurii*, and to the genus *Sporosarcina* (Bohácek et al., 1968b, Pechman et al., 1976; Stackebrandt and Woese, 1979). The data on the GC content and the chemical composition of the cell wall support these observations (Bohácek et al., 1968b; Schleifer and Kandler, 1970). There is no antigenic relationship of planococci to staphylococci and micrococci (Oeding, 1971). In the light of the above facts, therefore, the genus *Planococcus* should be placed into the family Bacillaceae as a separate genus.

Physiological and Biochemical Properties

Strains of *Planococcus citreus* and *P. kocurii* can grow in the absence of sodium chloride but can also tolerate as much as 10% sodium chloride. They grow in the range of 5–30°C. Growth at 37°C is variable. Their biochemical activities are rather restricted (Table 4). They are catalase positive and hydrolyze DNA and gelatin (variably). Acid is formed from glycerol and glucose (variably), but not from other sugars or sugar alcohols.

Planococci are negative for the following reactions: oxidase, methyl red, Voges-Proskauer, production of indole, H_2S, nitrate reduction, phenylalanine deaminase, lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase, urease, degradation of tyrosine, and hydrolysis of starch, esculin, and Tween 80. Assimilation of a

series of organic acids is variable or negative (Hao and Komagata, 1985).

Planococci are susceptible to lysozyme, chloramphenicol, erythromycin, novobiocin, oleandomycin, penicillin, and tetracycline (Jeffries, 1969; Kocur et al., 1970).

P. citreus was found to be one of the few marine bacteria that reproduced at a hydrostatic pressure of 20 to 40 MPa (Oppenheimer and ZoBell, 1952).

Identification

Planococci may be clearly separated from morphologically similar cocci by the characters listed in Table 4. They differ from the species of the genus *Marinococcus* by their ability to grow on media without NaCl added and by their inability to grow on peptone-yeast agar supplemented with 15% NaCl. *Sporosarcina halophila* differs from planococci in endospore formation, inability to grow on media containing 15% NaCl, esculin hydrolysis, and several chemotaxonomic characters. The two *Planococcus* species can be differentiated phenotypically only by their ability to grow on media containing 15% NaCl, which is positive only for *P. citreus*.

However, differentiation is also possible through their DNA base composition, zymogram, and protein pattern. They can also be separated serologically, as shown by Oeding (1971).

The Genus *Marinococcus*

The genus *Marinococcus* includes Grampositive, aerobic, and moderately halophilic cocci with *meso*-A_2pm acid in the cell wall. The genus was established by Hao et al. (1984) who transferred *Planococcus halophilus* reference is not an exact match Novitzki and Kushner (1976) into this genus and described the new species *Marinococcus albus*. Another new species, *M. hispanicus* has been isolated from a solar saltern (Marquez et al., 1990).

Habitat

Marinococci have only been isolated from marine environments, particularly from solar salterns (Ventosa et al., 1983; Marquez et al., 1990).

Isolation

No selective medium for the isolation of marinococci has been described. For their isolation, the salt complex medium of Ventosa et al. (1982) may be used.

Salt Complex Medium (Ventosa et al., 1982)

NaCl	8.1 g
MgCl$_2$	0.7 g
MgSO$_4$	0.96 g
CaCl$_2$	0.036 g
KCl	0.2 g
NaHCO$_3$	0.006 g
NaBr	0.026 g
Yeast extract	1.0 g
Proteose peptone No. 3	0.5 g
Glucose	0.1 g
Bacto-Agar (Difco)	20.0 g
Water	1 liter

Adjust to pH 7.5.

Cultivation

Marinococci can be cultivated on salt complex medium (Ventosa et al., 1982).

Maintenance and Preservation

Cultures of *Marinococcus* may be kept for 6 months at about 4°C on salt complex medium (Ventosa et al., 1982) if protected from drying. Cultures can be preserved in the freeze-dried state or in liquid nitrogen for long periods.

Genetic and Phylogenetic Relationships

The GC content of marinococci is in the range of 44.9–49.3 mol% (Hao et al. 1984; Marquez et al., 1990). DNA-DNA hybridization studies have not been performed within the genus.

Physiological and Biochemical Properties

Strains of *Marinococcus* species grow well in media with 20% sodium chloride, but not on media without salt. They grow in the range of 15–37°C. Like the planococci, the biochemical activity of the marinococci is restricted. They are catalase positive but oxidase production varies with species. *M. halophilus* and *M. hispanicus* may produce acid from glycerol and hydrolyze gelatin and esculin.

Marinococci are negative for the following reactions: acid from lactose, arabinose, galactose, and fructose; production of acetoin and indole; hydrolysis of starch and Tween 80; lysine and ornithine decarboxylases, arginine dihydrolase, phenylalanine deaminase, phosphatase, and growth on nutrient agar without sodium chloride.

Identification

Marinococci may be clearly separated from morphologically similar genera by the characteristics listed in Table 4. The three *Marinococcus* species can be differentiated by several phenotypic properties which are also listed in Table 4.

The Genus *Salinicoccus*

The genus *Salinicoccus* has been described quite recently (Ventosa et al., 1990). Its characteristics are based on only one strain which was isolated from a solar saltern near Alicante (Spain). The properties of this moderately halophilic nonsporeforming coccus are significantly different from all other hitherto described cocci. This justifies its recognition as a new species in a new genus.

The characteristics which distinguish *Salinicoccus roseus* from the phenotypically similar Gram-positive cocci are given in Table 4. There are no data on the ultrastructure, or the genetic and phylogenetic relationships of *S. roseus*.

The Genus *Filibacter*

A study of filamentous bacteria in sediments of lakes (Maiden, 1983) resulted in the isolation of an organism that resembled members of the genus *Vitreoscilla* in that it was a multi-cellular, filamentous, gliding bacterium that was not pigmented. Major differences in cytochrome and DNA base composition, isoprenoid quinone content, and sensitivity to actinomycin D seemed to indicate a more close relationship to the Flexibacteriaceae. As the organism did not resemble any previously described taxon of Flexibacteriaceae and on the basis of differences from both, it was proposed that the organism be placed in a new genus, *Filibacter*, with the type species *Filibacter limicola* Maiden and Jones, 1985, 375[VP] (Maiden and Jones, 1984).

Habitat and Isolation

Filibacter limicola has only been isolated once, from sediments of an eutrophic freshwater lake in the English Lake District, Blelham Tarn (Maiden, 1983). For isolation, sediment cores are sampled and treated according to Maiden and Jones (1984) as follows:

Isolation Procedure for *F. limicola*

> The top 1 to 2-cm layer of a sediment core is taken and diluted in filtered lake water. 10 µl of the sample are used for inoculation of plates containing the following medium:

MYP Medium (Maiden and Jones, 1984)

Peptone (Oxoid, L37)	0.1 g
Yeast extract (Difco)	0.01 g
K_2HPO_4	0.028 g
$MgCl_2 \cdot 6H_2O$	0.127 g
KNO_3	0.004 g
$(NH_4)SO_4$	0.06 g
$MnSO_4 \cdot 4H_2O$	0.008 g
Ferric citrate	0.006 g
Agar	10.0 g
Distilled water	1 liter

The pH of the medium is adjusted with potassium hydrogen carbonate to give a final pH of 7.0. The plates are incubated at 20°C for two weeks. They are periodically examined for growth of filaments through a dissecting microscope at a magnification of about 30×. *Filibacter* filaments may occur singly, in small groups, or in circular formations at some distance from the inoculation point. Purification may be done by excising small agar blocks with a single or a few attached filaments and transferring them to fresh medium. This procedure is repeated until pure cultures are obtained.

Cultivation

Pure cultures may be grown at about 20°C on nutrient agar (Oxoid CM 3), tryptone soya agar (TSA, Oxoid CM 131), or on the defined medium used for the isolation. Good growth is also obtained on media containing certain amino acids and vitamins as the sole source of carbon and nitrogen source (Maiden and Jones, 1984).

Cultures, incubated at 20°C, can be maintained on slants of TSA at 10°C for some weeks.

Cultural and Microscopic Appearance

Growth on solid media is characterized by spreading whorls of growth and spiral colonies which are not pigmented. The organisms exhibit gliding motility and grow in filaments of 8–150 µm where the individual cells are 3–30 µm long and about 1 µm wide. The cells are long rods that are either straight, or curved with rounded ends. Junctions between individual cells are marked by constrictions.

Capsules and sheaths are not formed but slime is excreted. Endospores and flagella as well as branching are not observed. Granules staining with sudan black were demonstrated but the test of Oste and Holt (1982) for poly-β-hydroxybutyrate is negative. Volutin granules are not observed. The cells stain Gram-negatively, although the cell wall is about 40 µm thick, lacks an outer membrane, and therefore shows the characteristics typical of a Gram-positive organism. Electron micrographs of thin sections showed a five-layered cell wall (Clausen et al., 1985).

Chemotaxonomy

Membrane fatty acids of *F. limicola* are dominated by anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$ components (Nichols et al., 1986). The cell wall of *Filibacter limicola* contains no diaminopimelic acid and MK-7 is the major quinone isoprenolog. Cytochromes of the *c* type were determined to predominate, with lesser amounts of *b*-type cytochromes (Maiden and Jones, 1984).

Genetic and Phylogenetic Relationship

The GC content of the DNA of the type strain is 44 mol% (T_m) (Maiden and Jones, 1984). Comparative 16S rRNA cataloging studies show that *Filibacter* clusters with the Gram-positive species *Bacillus globisporus*, with which it forms a branch within a grouping that comprises also *Caryophanon latum*, *Sporosarcina ureae*, *Planococcus citreus*, and other spherical sporeforming *Bacillus* species. Several phenotypic properties support the allocation of this organism to the family Bacillaceae.

Physiological and Biochemical Properties

The organism is strictly aerobic in the temperature range around 20°C, with 4°C as a minimum and no growth at 30°C. Catalase and oxidase are present. Casein or starch are not hydrolyzed whereas gelatin is. Sulfur globules are not deposited intracellularly in the presence of sulfide. Urease is present. Carbohydrates are not degraded. The only compounds which are weakly used as carbon sources in the presence of amino acids are acetate, lactate, butyrate, and glycerol.

A similarly narrow range of carbon sources can be found with spherical sporeforming *Bacillus* strains such as *B. pasteurii* or *B. sphaericus*, with strains of the genus *Caryophanon*, or with *Planococcus* strains.

Identification

Filibacter limicola is morphologically similar to *Vitreoscilla stercoraria* but not to the two other *Vitreoscilla* species described. Differences to *V. stercoraria* are listed in Table 5. It should be emphasized, however, that only one strain of each of the two species has been compared.

The Genus *Caryophanon*

The genus name *Caryophanon* (karyon [Greek] = nucleus; phaneros [Greek] = bright, conspicu-

Table 5. Differentiating properties of *Filibacter limicola* and *Vitreoscilla stercoraria*.

Character	*F. limicola*	*V. stercoraria*
Strict aerobe	+	+
Catalase	+	+
Oxidase	+	−
Growth at 4°C	+	−
Growth at 30°C	−	+
Hydrolysis of gelatin	+	−

Adapted from Maiden and Jones (1984).

ous, that which has a conspicuous nucleus) is a misnomer, arising from the original false conclusion that the cross-walls, nuclear material, and cytoplasm seen in stained trichomes was a nucleus. The true nature of the trichomes was shown by improved cytological techniques (Pringsheim and Robinow, 1947).

Historically, *C. latum* played a significant role in the development of bacterial cytology in the 1940s and 1950s: Because of the large size of its trichomes and its numerous stainable "nuclei," *Caryophanon* provided an ideal model in the "great mitosis" debate (Tuffery, 1955).

Today, two species are recognized, *Caryophanon latum* Peshkoff 1939, 244[AL] and *Caryophanon tenue* (Peshkoff) Trentini 1988, 220[VP] (effective publication: Trentini 1986, 1259). Affiliation of the genus to already known, specific taxa was always difficult. Trichome-forming bacteria are a diverse group of organisms that have as a common property an outer cell wall that holds together the individual cells of the trichome.

In the seventh edition of *Bergey's Manual of Determinative Bacteriology* (1957) the order Caryophanales was described to include trichome-forming organisms comprising three families: Caryophanaceae, Oscillospiraceae (sporeforming organisms, not in pure culture) and Arthromitaceae. Later, in the eighth edition of *Bergey's Manual* (1974), *Caryophanon* was listed under Part 16: Gram-Positive, Asporogenous Rod-shaped Bacteria: Genus of Uncertain Affiliation. In *Bergey's Manual of Systematic Bacteriology, Caryophanon* was placed into Section 14: Regular, Nonsporing, Gram-positive Rods. However, recent rRNA sequencing studies have revealed a close relationship of *Caryophanon* to the aerobic sporeforming bacteria (Stackebrandt et al., 1987).

Habitat

Caryophanon latum was first isolated in 1937 and *C. tenue* in 1938 from fresh cow manure (Peshkov, 1939). In most of the successive studies, cattle manure was used as the primary source of these organisms (Gershenfeld and Lam, 1953; Kele, 1970; Moran and Witter, 1976; Peshkov and Marek, 1973; Pringsheim and Robinow, 1947; Provost and Doetsch, 1962; Smith and Trentini, 1972; Trentini and Machen, 1973; reference is not an exact match Tuffery, 1955; Weeks and Kelley, 1958). Reports that *C. latum* has been found in or on other sources such as sewage (Tuffery, 1953), the oral cavity of dogs (Saphir and Carter, 1976), or decaying *Pleurotus* on the stump of a tree (R. E. Buchanan, unpublished observations) could not be verified. Other findings that *Caryophanon* occurs associated with manure of

other animals (Kele, 1970; Trentini and Machen, 1973; reference is not an exact match Dean, 1973) may be doubted because of possible contamination of these sources with cattle manure.

Most samples taken from rumen fluid, cattle saliva, teeth scrapings, anal swabs, or rectal and intestinal samples from slaughtered cattle were negative for *Caryophanon*. Also, *Caryophanon* was not found in aseptically sampled cattle manure. It may, therefore, be concluded that these bacteria are not residents of the bovine digestive tract (Pringsheim and Robinow, 1947; Trentini and Machen, 1973; Kele, 1970; Dean, 1963).

Isolations of *Caryophanon* are easiest from 1- to 2-day-old droppings, whereas old field-dried cattle manure was usually negative for these organisms. Although the natural habitat of *Caryophanon* is not definitely known, it is assumed that these bacteria are secondary contaminants of cattle manure, dispersed to new droppings by air, by insects, or by the cattle itself.

Enrichment and Isolation

Caryophanon strains can be isolated only after an enrichment step. Good enrichment results are regularly obtained with fresh droppings from the barn gutters found with stanchioned cattle or with pasture manure after 1 to 2 days of field-aging as long as little or no rain has fallen during that time and the temperature has not fallen below 0°C.

Enrichment Procedure (Pringsheim and Robinow, 1947; Smith and Trentini, 1972)

Cow dung about two days old is collected from pastures and brought to the laboratory as soon as possible. Samples of 600–800 ml are distributed into beakers and thoroughly mixed with distilled water so that the surface remains covered by a thin layer of water. The beakers are covered with aluminum foil. After 16–24 h of incubation at room temperature, top slurry material is viewed under the microscope for the typical, actively motile *Caryophanon* trichomes and trichome chains.

Attempts at isolation should be made only on samples in which, at a magnification of 400×, *C. latum* occurs in numbers of at least three to four trichomes or trichome-chains per microscopic field. More concentrated samples with up to 20–30 trichomes per field may also be observed.

For isolation, plates may be inoculated directly from this surface material. More enhanced isolation may be achieved by removing as far as possible larger and smaller particles from the slurry such as plant material, protozoa, bacteria, and bacterial and fungal spores. It is necessary to combine several filtration and centrifugation steps:

Isolation Procedure

Skim off the top slurry material from samples with high numbers of trichomes and filter it through coarse filter paper (e.g.) coffee filter paper) to remove large particles (very small pores would soon be clogged by the very fine dung particles). Rinse the solid matter trapped by the filter paper with about 10 ml distilled water and filter again by squeezing it gently.

Centrifuge the combined filtrate using a rotor with approximately r = 15 cm at 1500 rpm. Under these conditions the relatively large trichomes will sediment in about 5 min. Discard the supernatant which includes a large proportion of the fine material. Resuspend the pellet in nutrient broth and repeat the filtration, centrifugation, and resuspension steps once or more.

By applying vacuum, filter the suspension through a membrane filter of about 8-μm pore diameter while adding repeatedly about 20 ml nutrient broth to a total amount of 400 ml. Take care to keep the filter moist throughout this procedure to prevent injury to the trichomes from drying. Remove the membrane filter and agitate it thoroughly along with 2 ml nutrient broth in a flask to wash off the adherent trichomes.

From the final suspension, prepare 10^{-2} to 10^{-5} dilutions and spread 0.1 ml of each dilution on cow dung agar plates containing 80 μg/ml streptomycin.

Cow Dung Agar (Smith and Trentini, 1972)

Cow dung	250 ml
Distilled water	750 ml
Agar	15 g

Mix fresh cow dung with distilled water for 2–3 min in a laboratory mixer to break up large dung particles and ensure thorough dispersion. Add additional water up to I liter and sterilize the medium for 20 min at 121°C. 10 ml of filter-sterilized streptomycin solution (0.8 g per 100 ml) is added after heat sterilization of the medium.

After incubation at room temperature for 48 h, typical colonies of *Caryophanon* colonies may develop. To obtain pure cultures, suspended material from single colonies is repeatedly restreaked onto cow dung agar with or without streptomycin.

Cultivation

Growth of *Caryophanon* strains is good on cow dung agar described above without the addition of streptomycin. Several semisynthetic media have been described for *C. latum* (Pringsheim and Robinow, 1947; Provost and Doetsch, 1962; Kele and McCoy, 1971; Smith and Trentini, 1973). However, they are all not as good as cow dung agar with respect to doubling time and maintenance of the original morphology.

Cultures usually grow best at room temperature or 25°C at a pH of 7.8–8.5. Moran and Whitter (1976), however, stated that 35°C is the optimal temperature for growth of *C. latum*, based upon the rate of change in colony diameter on a given medium.

Maintenance and Preservation

Active cultures of both *Caryophanon* species may be kept at 4–10°C on agar slants for more than 4–6 weeks if protected from drying. Cultures are readily preserved by standard techniques such as freezing in liquid nitrogen or lyophilization.

Cultural and Microscopic Appearance

Colonies of *C. latum* are pale yellow, about 1.5 mm in diameter, opaque, granular, lobate, and show a glistening surface after growth for 48 h on cow dung agar or cow dung agar containing 0.5% lactalbumin hydrolysate at room temperature. Colonies of *C. tenue* are similar to those of *C. latum* in overall appearance, except that they are only 0.5–1.0 mm in diameter and less irregular in shape under the same conditions.

Trichomes of *C. latum* are about 3 μm in width, and 10–20 μm in length. They are straight or slightly curved. Trichome chains may be formed especially in enrichment cultures. They contain two to six trichomes. Individual cells within a trichome are wider than they are long, and several growing septa at various stages of closure can be observed at the same time in each cell.

The size and shape of the organism vary with the culture conditions. As growth proceeds, transverse fissions may give rise to even-shorter, and even spherical, forms. Size frequently diminishes during laboratory cultivation, so that eventually most of the trichomes in a culture may not exceed 1 μm in diameter.

Trichomes of *C. tenue* are about 1.5 μm in width, 10–20 μm in length, straight or slightly curved, and are composed of fewer cells than the trichomes of *C. latum*. Individual cells within a trichome are longer than wide. Only one cross septum can be observed per single cell (Peshkov and Marek, 1973), which may be explained by the much slower growth of *C. tenue* in comparison to *C. latum*.

Caryophanon trichomes are Gram-positive, peritrichously flagellated, and show no branching. Their ends are round or slightly tapered. Under optimal growth conditions, individual cells exhibit a disk-like shape. Cross walls between cells show as dark lines, partly complete, partly developing by ingrowth from the external wall. Endospores, sheaths, and capsules are not formed.

In old cultures or under growth conditions which lead to extensive lysis, a small number of cell units is preserved as round bodies (spheroids). The significance of the spheroids is unknown but it has been suggested that they are part of a "life cycle" (Peshkov, 1939). Under good growth conditions, the spheroids are said to be able to grow into normal trichomes. It should be noted here that almost all investigations have been carried out with *Caryophanon latum*, the morphology of which is strikingly sensitive to growth conditions.

Morphology and motility of *Caryophanon* are difficult to maintain. Both characteristics are reported to be best preserved when 0.5 to 1% lactalbumin hydrolysate is added to cow dung medium. The most authentic morphology and motility of the trichomes, though, can still be observed in enrichment cultures. Optimal growth conditions that will maintain natural trichome morphology as well as mass or colony diameter increase in pure cultures have still to be developed.

Ultrastructure

In cells of *C. latum*, one or two superficial wall layers containing protein are present (Trentini and Gilleland, 1974). Mesosomes, nucleidosomes, and analogs of mitochondria were described by Shadrina et al. (1982).

Chemotaxonomy

Diaminopimelic acid is absent in *C. latum* (Becker et al., 1967). The peptidoglycan of the cell wall is composed of glutamic acid, alanine, lysine, and muramic acid in a molar ratio of $2:2:1:1$. Wall material is sensitive to lysozyme (Trentini and Murray, 1975). Teichoic acids and *O*-acetyl groups are absent (W. Trentini, unpublished observations).

Genetic and Phylogenetic Relationships

Thirty-six strains of *C. latum* isolated from various geographic regions exhibit GC contents of 44.0–45.6 mol% DNA-DNA hybridization studies reveal—with a homology of 78–92%—a high homogeneity among the strains (Adcock et al., 1976). The genome size is $1,100–1,200 \times 10^6$ dalton.

Three strains of *C. tenue* examined for GC content exhibit values of 41.2–41.6 mol% (Adcock et al. 1976). The genome size is $900–1,000 \times 10^6$ dalton. DNA-DNA reassociation studies between *C. tenue* and *C. latum* revealed low homology values of 13–30%.

A close phylogenetic relationship of *Caryophanon* to members of the genus *Bacillus*, in particular to the spherical sporeforming *Bacillus* species, has been described by Stackebrandt et al. (1987). In a dendrogram based on rRNA sequencing studies, the closest relative of *Caryophanon* was *Bacillus sphaericus*. In the same cluster were found *Planococcus citreus*, *Filibacter limicola*, and *Sporosarcina ureae*, as

well as other spherical sporeforming *Bacillus* species.

Physiological and Biochemical Properties

Studies on physiological and biochemical properties of the two *Caryophanon* species have been published mainly by Pringsheim and Robinow (1947), Provost and Doetsch (1962), and Adcock et al. (1976).

Both species are strict aerobes. Catalase is formed. The cytochrome-oxidase reaction is negative. Nutrition is chemoorganotrophic. Starch, gelatin, casein, and cellulose are not hydrolyzed, and indole is not produced. Acetate is used as a major carbon source. More recent studies (Rowenhagen, 1987) revealed that a number of other fatty acids (e.g.) butyrate, valerate, capronate, stearate, methylpropionate, and 2-methylbutyrate) are oxidized by most *Caryophanon* strains. Biotin has been found to be essential for growth, and thiamine seems to be stimulatory.

Strains of *C. latum* were shown not to degrade uric acid or urea and not to utilize glucose and other sugars. Poly-β-hydroxybutyrate is used as a carbon source. Tributyrin is only very weakly hydrolyzed. Nitrates are not reduced to nitrites. These organisms grow at 10 and 37°C but not at 45°C and at pH values between 6 and 8, with an optimum at pH 7.6–8 (Weeks and Kelley, 1958).

For both species, no information has been published on the major metabolic pathways, genetics, or antigenic structure. In almost all cultures with an unbalanced nutrition (e.g., older cultures) poly-β-hydroxybutyrate accumulates. *Caryophanon* strains are resistant to streptomycin, nalidixic acid, several sulfa drugs, and polymyxin B.

Drozd et al. (1987) described a case of concrete corrosion caused by a *Caryophanon* species.

Identification

The genus and the two recognized species can only be identified mainly through their morphological properties (Table 6).

A species-specific phage for *C. tenue* has been described (Peshkov et al., 1973; Peshkov et al., 1966).

Table 6. Differentiating properties of *C. latum* and *C. tenue*.

Character	C. latum	C. tenue
Trichome width (μm)	2.8–3.2	1.4–2.0
Number of cross septa/cell unit	several	1
GC content (mol%)	44.0–45.6	41.2–41.6
Genome size (×10⁶ dalton)	1,100–1,200	900–1,000

Adapted from Trentini (1986).

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CHAPTER 1.2.20

An Introduction to the Family Clostridiaceae

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Introduction

The pre-16S rRNA sequence definition of the genus *Clostridium* was—non-sulfate-reducing sporeformer relying obligately on anaerobic energy metabolism and with a Gram-positive type cell wall (Hippe et al., 1992). Our view of bacterial classification has greatly changed in the past 10 years. We now define the overall taxonomic structure of the prokaryotes, for the most part, on the basis of relationships revealed by comparison of 16S rRNA gene sequences. This has lead to transferring many former clostridial species to novel genera and to a more narrow definition of the genus *Clostridium* (further referred to as *Clostridium* sensu stricto), encompassing much fewer species but including the majority of the medically important species.

Phylogeny

The recent release of the second edition of *Bergey's Manual of Systematic Bacteriology* takes the bold step of proposing a taxonomic structure for all validly described prokaryotes on the basis of a 16S rRNA gene sequence phylogeny. This taxonomic structure in the form of Bergey's Taxonomic Outline is available {online}. Such a structure provides a backbone for a classification system that can be added to as new taxa are discovered and described or can be used to identify taxa that are misplaced. The family Clostridiaceae as defined by the taxonomic outline of *Bergey's Manual of Systematic Bacteriology* contains as its core the genus *Clostridium* (sensu stricto), as well as *Acetivibrio*, *Acidaminobacter*, *Alkaliphilus*, *Anaerobacter*, *Caloramator*, *Caloranaerobacter*, *Coprobacillus*, *Dorea*, *Natronincola*, *Oxobacter*, *Sarcina*, *Sporobacter*, *Thermobrachium*, *Thermohalobacter* and *Tindallia*. The previous inclusion of these additional genera in a family Clostridiaceae is based for the most part on the fact that the type species of these genera are in many cases phylogenetically related to misclassified species of the genus *Clostridium*. However

with the exception of *Anaerobacter*, *Caloramator*, *Oxobacter*, *Sarcina* and *Thermobrachium*, these genera fall outside the radiation of what can be considered the true family Clostridiaceae and are now regarded as belonging to other families within the low G + C Gram-positive phylum. This misclassification is the result of well-known problems of the current taxonomic structure of the traditional genus *Clostridium*. The phylogenetic analysis of Collins et al. (1994) was the first large-scale comparison of 16S rRNA gene sequences of species of the genus *Clostridium* and related taxa. The fact that the species of the genus *Clostridium* did not form a monophyletic group has been shown in a number of studies in which small groups of *Clostridium* species had been compared as far back as 1981 (Tanner et al., 1981; Hutson et al., 1993; Lawson et al., 1993; Rainey and Stackebrandt, 1993a; Rainey et al., 1993b; Willems and Collins, 1994). The comparative study of Collins et al. (1994) and subsequent studies (Stackebrandt and Rainey, 1997; Stackebrandt et al., 1999) can be used to conclude that more than half of the species currently assigned to the genus *Clostridium* are in fact not closely related to the type species of the genus, *C. butryicum,* and from a phylogenetic standpoint should not be included in a newly defined genus *Clostridium*. This extensive genetic diversity of the genus *Clostridium* had been shown using 23S rRNA:DNA hybridization studies back in 1975 by Johnson and Francis (1975), but it was the 16S rRNA gene sequence approach that revealed the actual phylogenetic relationships between the species of this genus and other genera.

There are two main issues that affect the taxonomy of the genus *Clostridium* and related taxa and their allocation to a genus or family within the 16S rRNA-based phylogenetic structure of the Bacteria. Firstly, the genus *Clostridium* current comprises 152 validly described species (see the List of Bacterial Names with Standing in Nomenclature (http://www.bacterio.net). However, on the basis of 16S rRNA gene sequence analyses, only 73 of these validly described species fall within the radiation of the

type species of the genus *Clostridium*, *C. butyricum*, and the cluster I as defined by Collins et al. (1994) (Table 1; Fig. 1). We will refer hereafter to this cluster I as the genus *Clostridium* sensu stricto. The remaining species fall into various phylogenetic clusters throughout the low G + C Gram-positive phylum (Table 2). These 16S rRNA gene sequence based clusters (Collins et al., 1994) can be correlated with the Bergey's Taxonomic Outline to provide a family or proposed family placement for each of the validly described *Clostridium* species (Table 2). The second issue related to the fact that the genus *Clostridium* (sensu stricto, cluster I) has members of other valid genera and in two cases the type species of

a validly described genus clustering within its radiation. The genera that have species that fall within the genus *Clostridium* (sensu stricto) are *Anaerobacter* (*A. polyendosporus*), *Eubacterium* (including *E. moniliforme* and *E. tarantellae*) and *Sarcina* (*S. ventriculi* and *S. maxima*). The position of these taxa within the genus *Clostridium* sensu stricto is shown in Fig. 1.

In the case of the *Eubacterium* species these taxa are misclassified and were placed in the genus *Eubacterium* on the basis of the characteristic that endospore formation was not observed. The genus *Anaerobacter* was described before 16S rRNA gene sequences were available, but this organism does form endospores and has all of the characteristics that would warrant its transfer to the genus *Clostridium* sensu stricto. As first reported by Willems and Collins (1994), both species of the genus *Sarcina* fall within the genus *Clostridium* sensu stricto and in fact are more closely related to the type species of the genus *Clostridium*, *C. butyricum*, than are many of the other *Clostridium* species. This situation is more complicated than that of the *Eubacterium* and *Anaerobacter* species that can be reclassified, in that the genus *Sarcina* was in fact described before the genus *Clostridium* and so has taxonomic priority. However, considering the importance of many of the species of the genus *Clostridium* in animal and human health, the priority is overlooked in this case and both genera retain their original names.

Both of these situations that contribute to the taxonomic disorder of the genus *Clostridium* are due to classification of new isolates for the most part on the basis of phenotypic characteristics, e.g., Gram-positive staining, anaerobic growth, and endospore formation. If a Gram-type or Gram-positive staining organism formed endospores and grew anaerobically, it was added to the genus *Clostridium* and designated a new species in earlier times on the basis of some unique physiological property. This system has continued even when 16S rRNA gene sequences have been determined for new isolates, and it is clear that the new isolates are not members of the genus *Clostridium* in that they fall outside of cluster I (Collins et al., 1994) and are more closely related to other genera than to the type species of the genus *Clostridium* sensu stricto. In many cases these new isolates, although not showing a close phylogenetic relationship to cluster I *Clostridium* species do group with misclassified *Clostridium* species that in fact belong to families other than the family Clostridiaceae. A good example of this is the *Clostridium* species of cluster XIVa. Many of these are polysaccharolytic, cluster together on the basis of 16S rRNA gene sequence analyses, but are unrelated to the clustered of *Clostridium* sensu stricto species. As

Table 1. The species belonging to the genus *Clostridium* sensu stricto (cluster I).[a]

Cluster I species	Cluster I species
C. absonum	*C. lacusfryxellense*
C. acetireducens	*C. lentoputrescens*
C. acetobutylicum	*C. limosum*
C. acidisoli	*C. ljungdahlii*
C. akagii	*C. magnum*
C. algidicarnis	*C. malenominatum*
C. argentinense	*C. novyi*
C. aurantibutyricum	*C. oceanicum*
C. baratii	*C. paraperfringens*
C. beijerinckii	*C. paraputrificum*
C. botulinum	*C. pascui*
C. bowmanii	*C. pasteurianum*
C. butyricum	*C. peptidivorans*
C. cadaveris	*C. perenne*
C. carnis	*C. perfringens*
C. celatum	*C. pfennigii*
C. cellulovorans	*C. proteolyticum*
C. chartatabidum	*C. puniceum*
C. chauvoei	*C. putrefaciens*
C. cochlearium	*C. putrificum*
C. colicanis	*C. quinii*
C. collagenovorans	*C. roseum*
C. cylindrosporum	*C. saccharobutylicum*
C. disporicum	*C. saccharoperbutylacetonicum*
C. estertheticum subsp. *estertheticum*	*C. sardiniense*
C. estertheticum subsp. *laramiense*	*C. sartagoforme*
C. fallax	*C. scatologenes*
C. frigidicarnis	*C. septicum*
C. frigoris	*C. sporogenes*
C. gasigenes	*C. subterminale*
C. grantii	*C. tertium*
C. haemolyticum	*C. tetani*
C. histolyticum	*C. tetanomorphum*
C. homopropionicum	*C. thermobutyricum*
C. intestinale	*C. thermopalmarium*
C. isatidis	*C. tyrobutyricum*
C. kluyveri	*C. vincentii*

[a]According to Collins et al. (1994) and subsequent publications describing new *Clostridium* species.
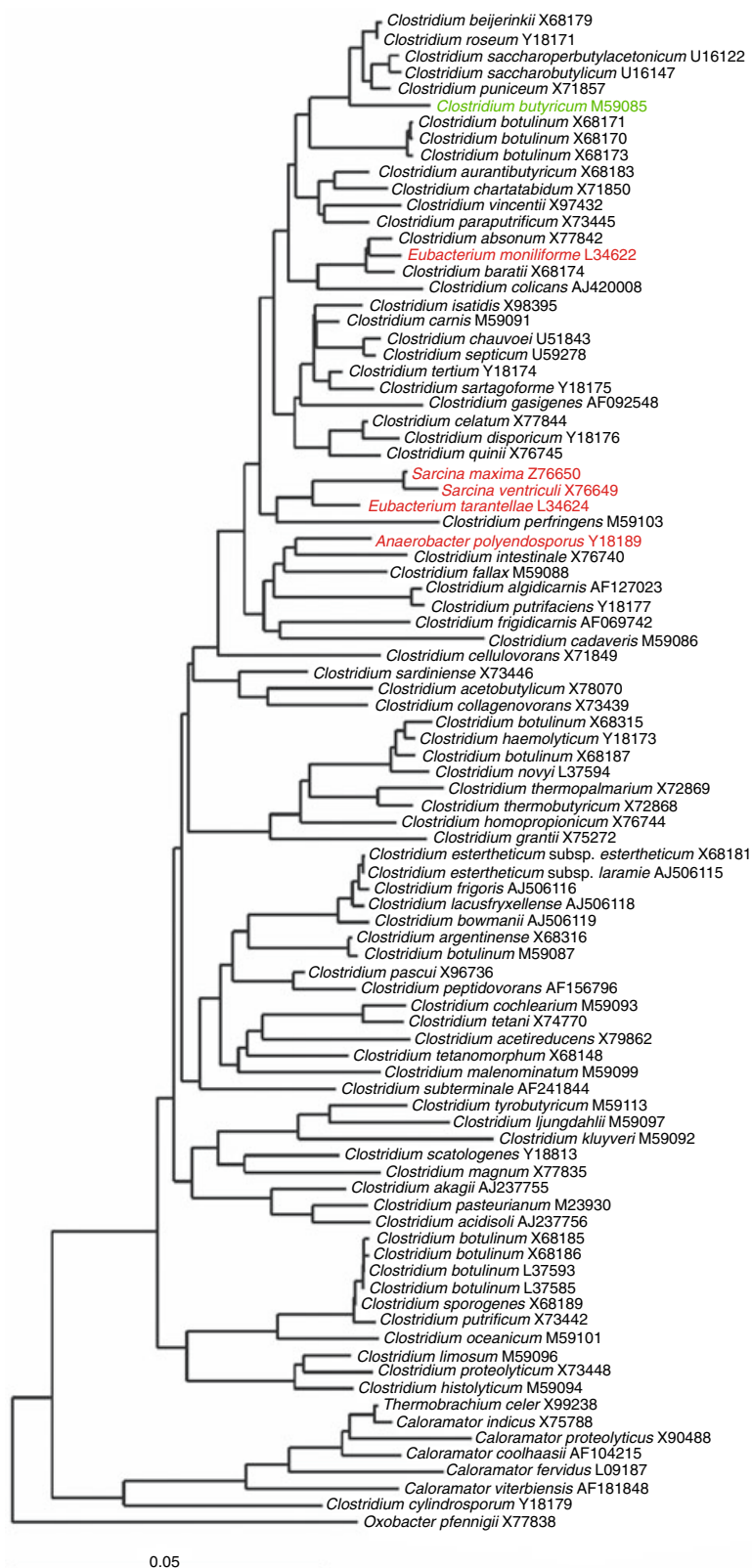
Fig. 1. Phylogenetic dendrogram showing the relationships between the genera and species of the family Clostridiaceae. The phylogenetic dendrogram was reconstructed from evolutionary distances by the neighbor-joining method using the PHYLIP software package (Felsenstein, 1993). The scale bar represents 5 inferred nucleotide changes per 100 nucleotides. Taxa in red represent valid genera that fall within the radiation of the genus *Clostridium* sensu stricto. The type species of the genus *Clostridium*, i.e., *Clostridium butyricum*, is indicated in green.

Table 2. The species of the genus *Clostridium* that fall outside of the genus (cluster I).[a]

Cluster III represents a new family	*C. aminophilum*
C. aldrichii	*C. aminovalericum*
C. cellobioparum	*C. celerecrescens*
C. cellulolyticum	*C. clostridioforme*
C. hungatei	*C. coccoides*
C. josui	*C. fimetarium*
C. papyrosolvens	*C. hathewayi*
C. stercorarium	*C. herbivorans*
C. termitidis	*C. hylemonae*
C. thermocellum	*C. indolis*
C. thermolacticum	*C. leptum*
Acetivibrio cellulolyticus	*C. litorale*
Bacteroides cellulosolvens	*C. lituseburense*
Cluster IV is within the proposed family "Lachnospiraceae"	*C. methoxybenzovorans*
C. cellulose	*C. methylpentosum*
C. leptum	*C. nexile*
C. orbiscindens	*C. oroticum*
C. sporosphaeroides	*C. phytofermentans*
C. viride	*C. polysaccharolyticum*
Ruminococcus bromii	*C. populeti*
Cluster XIa is within the proposed "Peptostreptococcaceae"	*C. proteoclasticum*
C. bifermentans	*C. saccharolyticum*
C. difficile	*C. scindens*
C. ghonii	*C. sphenoides*
C. glycolicum	*C. sporosphaeroides*
C. hiranonis	*C. symbiosum*
C. irregulare	*C. xylanolyticum*
C. litorale	*C. xylanovorans*
C. lituseburense	**Cluster XIVb is within the proposed "Lachnospiraceae"**
C. mangenotii	*C. colinum*
C. mayombei	*C. lactatifermentans*
C. paradoxum	*C. lentocellum*
C. sordellii	*C. neopropionicum*
C. sticklandii	*C. piliforme*
C. thermoalcaliphilum	*C. propionicum*
Eubacterium acidaminophilum	*Eubacterium yurii*
Peptostreptococcus anaerobius	"*Epulopiscium*" species
Cluster XIb is within the proposed "Peptostreptococcaceae"	**Cluster XVI is a new family**
C. felsineum	*C. innocuum*
C. formicaceticum	*Eubacterium biforme*
C. halophilum	*Eubacterium cylindroids*
Filifactor villosus	*Eubacterium dolichum*
Cluster XII is within the proposed "Peptostreptococcaceae"	*Eubacterium tortuosum*
C. acidurici	*Eubacterium pleomorphus*
C. hastiforme	**Cluster XVIII is a new family**
C. purinilyticum	*C. cocleatum*
C. ultunense	*C. ramosum*
Eubacterium angustum	*C. spiroforme*
Eubacterium thermomarinus	**Cluster XIX is within the proposed "Fusobacteriaceae"**
Tissierella species	*C. rectum*
Cluster XIVa is within the proposed "Lachnospiraceae"	*Fusobacterium nucleatum*
C. aerotolerans	*Leptotrichia buccalis*
C. algidixylanolyticum	*Propionigenium modestum*

[a]As defined by Collins et al. (1994). The family designations of these species based on the cluster placement within the Bergey's Taxonomic outline is provided as well as examples of valid genera within these clusters.

new species are isolated and described in this cluster XIVa, they have been assigned to the genus *Clostridium*, further adding to the taxonomic disorder of the genus.

The *Clostridium* sensu stricto species, the group considered to be the true genus *Clostridium* owing to the presence of the type species *C. butyricum* within this group, represent a phylogenetically diverse group with 16S rRNA gene sequence similarities in the range 92% (the most distant relationships) to >99% (closely related species; Table 1). Within cluster I, the species *C. botulinum* is found dispersed throughout, as shown previously by Hutson et al. (1993) (Fig. 1). The type strain of *C. botulinum*, ATCC 25763T (L37585) groups with *C. sporogenes* and *C. putrificum* (invalid name) and is rather distantly related to other strains bearing the *C. botulinum* species epithet (Fig. 1). At the level of the family Clostridiaceae, including the genera *Caloramator*, *Oxobacter* and *Thermobranchium*, which fall outside of genus *Clostridium* sensu stricto, the 16S rRNA gene sequence similarities range from 89% to >99%. Note that in previous studies, including Collins et al. (1994), the species *C. histolyticum*, *C. limosum* and *C. proteolyticum* grouped outside of this cluster and were assigned to cluster II. As new species have been added to these and the outgroup clusters, the topology of the reconstructed trees have changed somewhat and the distinct separation of the cluster II species from those of cluster I (*Clostridium* sensu stricto) is becoming less clear. There is still however a clear separation of clusters I and II from the other genera of the family that lie outside of cluster I (Fig. 1). Stackebrandt and Rainey (1997) divided cluster I into subclusters Ia through Ii on the basis of the phylogenetic grouping evident within cluster I at the time of that analysis. As new species have been added to cluster I the topology of the subclusters has changed in some cases, and so it seems that redefining the designations of subclusters at this time does not improve the taxonomy of the genus *Clostridium*.

The genera that group outside of *Clostridium* sensu stricto but from a 16S rRNA gene sequence phylogeny perspective fall within the family Clostridiaceae have either been *Clostridium* species transferred to new genera (in the case of *Clostridium fervidum* going to the genus *Caloramator* as *Caloramator fervidus* [Collins et al., 1994] and *Clostridium pfennigii* transferred to *Oxobacter* as *Oxobacter pfennigii* [Collins et al., 1994]) or to newly described genera in the case of *Thermobrachium,* a very close relative of *Caloramator* (Engle et al., 1996).

Other species of the genus *Clostridium* have been transferred from the genus to new genera on the basis of 16S rRNA gene sequence com-

parisons that have shown them to fall outside the radiation of the genus *Clostridium* sensu stricto and outside the family Clostridiaceae. These include *Clostridium* barkeri → *Eubacterium barkeri* (Collins et al., 1994), *Clostridium bryantii* → *Syntrophospora bryantii* (Zhao et al., 1990), *Clostridium durum* → *Paenibacillus durus* (Collins et al., 1994), *Clostridium hydroxybenzoicum* → *Sedimentibacter hydroxybenzoicus* (Breitenstein et al., 2002), *Clostridium lortetii* → *Sporohalobacter loretii* (Oren et al., 1988), *Clostridium oxalicum* → *Oxalophagus oxalicus* (Collins et al., 1994), *Clostridium quercicolum* → *Dendrosporobacter quercicolus* (Strompl et al., 2000), *Clostridium thermaceticum* → *Moorella thermacetica* (Collins et al., 1994), *Clostridium thermautotrophicum* → *Moorella thermautotrophica* (Collins et al., 1994), *Clostridium thermocopriae* → *Thermoanaerobacter thermocopriae* (Collins et al., 1994), *Clostridium thermohydrosulfuricum* → *Thermoanaerobacter thermohydrosulfuricus* (Lee et al., 1993), *Clostridium thermosaccharolyticum* → *Thermoanaerobacterium thermosaccharolyticum* (Collins et al., 1994), *Clostridium thermosulfurigenes* → *Thermoanaerobacterium thermosulfurigenes* (Lee et al., 1993), and *Clostridium* villosum → *Filifactor villosus* (Collins et al., 1994).

As indicated above, the majority of the species currently assigned to the genus *Clostridium* fall outside the genus as defined by Collins et al. (1994) and outside the family Clostridiaceae. Table 2 indicates the families to which these *Clostridium* species, which we should consider as incertae sedis at this time, might be allocated on the basis of their association with other genera and species. Collins et al. (1994) not only designated these species to clusters II through XVIII but indicated that they might represent taxa at family levels, families that they designated 1 through 13. The taxonomic outline as presented by Bergey's Manual® allows for the placement of each of these misclassified *Clostridium* species in a family. This provides the basis for a reclassification of these species to existing genera within these families or the description of new genera. Table 2 shows the family affiliation for each of the *Clostridium* species that fall outside of the genus *Clostridium* sensu stricto (cluster I).

Habitats

The habitats and ecology of the type species of *Clostridium* (Cato et al., 1986), *Clostridium butyricum*, is representative for many species in this family. *Clostridium butyricum* is a mesophilic, Gram-positive staining, sporeforming anaerobe found in soil, sediments, the rumen,

and even in the decaying heartwood of living trees. The anaerobic nature of this bacterium (or a close relative) was first noted by Pasteur in his description of "vibrion butyrique" (Pasteur, 1861). Like a number of other mesophilic clostridia and related bacteria found in soil, *C. butyricum* has been recovered from wounds and abscesses, but it is not a pathogen in the same sense as *Clostridium perfringens* or *Clostridium tetani*. *Clostridium butyricum* can fix nitrogen, as do a number of other clostridia. The ability to fix nitrogen is probably more widespread in the Clostridiaceae than currently appreciated. The metabolism of *C. butyricum* is primarily saccharolytic, as it is for the majority of the described species of Clostridiaceae.

Because of the nature of forming spores, generally clostridia and related species can be isolated from nearly every environment including melted snow or ice from Antarctica, dry desert sand, alkaline and acidic hot spring water and mud, mesobiotic and psychrobiotic river and lake water or sediments, and intestinal tracts of animals and insects. Thus clostridia as a group are truly ubiquitous. Even pathogenic clostridia including *C. botulinum* and *C. tetani* can be isolated from nearly every aerobic soil sample and dust, a reason that both are still important pathogens despite available immunization against *C. tetani*. Also, clostridia have been isolated from feces since their spores can stay viable when passing through intestinal tracts.

However, when dealing with habitat questions one has to keep in mind that because of the presence of nongrowing spores, isolation of clostridia and related bacteria from an environment or the presence of a corresponding 16S rRNA gene sequence, does not indicate that the isolates or the corresponding bacteria grew or were able to grow and multiply in that environment. A first estimate of whether a specific species could grow and multiply in a given environment can be made by comparing the known or freshly tested physiological properties of the species or isolates in question and the environmental parameters of the sampling place. However, a word of caution has to be made here, in that frequently bulk measurements for environmental samples do not reflect absence of possible growth conditions available in microniches within the given site. Conditions in such microniches can provide on small scales, sometimes only temporarily, significantly different growth conditions (i.e., substrate concentrations, pH, temperature, and other nutrients) than those represented by bulk measurements. One well-documented example is that the high respiratory activity of one or more aerobic bacteria can provide an anaerobic microniche in which obligate anaerobic bacteria could grow, e.g., the oxygen

sensitive N_2-fixing clostridia in aerobic *Bacillus* agar surface colonies as described by Postgate (1974) and Line and Loutit (1973) more than 30 years ago. One way to test whether the bacteria are growing is to compare enumerations (e.g., most probable numbers [MPNs], with extended incubation temperatures to allow spores to germinate) in pasteurized (or in case of thermophiles, heated for 1–5 min at 100ºC) and in untreated samples. Another example is the fast-growing *Thermobrachium celere* from various mesobiotic environments. The doubling times of strains from mesobiotic environments are 10–20 min, whereas the doubling times from strains from hot springs have doubling times above 30 min. *Thermobrachium* is also a moderate alkaliphile (alkalithermophile) that was isolated from environments with bulk pH values below pH 5.0 (Engle et al., 1996; M. Engle and J. Wiegel, unpublished data).

Isolation and Cultivation

The underlying phenotype common to all of the members of the family Clostridiaceae is the anaerobic phenotype. So, anaerobic technique is essential for the enrichment, isolation and culture of members of this group (see Ljungdahl and Wiegel [1986], Hippe et al. [1992], and Tanner [2002]).

Anaerobic Culture Technique

The recommended procedures for current anaerobic technique were developed in the laboratory of Ralph S. Wolfe during the mid-1970s and are generically referred to as "the Balch technique." The Balch technique is based on the Hungate technique, used for many of the seminal advancements in anaerobic microbiology in the 1960s and still used today. For example, roll tubes are still one of the easier ways to manipulate anaerobes on agar surfaces or in agar using the agar-shake-roll tube method (Ljungdahl and Wiegel, 1986; Tanner, 2002). These techniques use specialized user-friendly glassware and equipment to easily manipulate anaerobes or any microorganism requiring a defined gas phase. These procedures will be the focus of the discussion here. Some of the key methodological references for anaerobic microbiology are given in Table 3. Various laboratories working with anaerobes have developed their own modifications depending on preferences or needs.

Simpler approaches to anaerobic microbiology are covered in the literature (Willis, 1969; Hippe et al., 1992), much of it targeted towards medically important clostridia (Sutter et al., 1985) that are in general less sensitive to air or

Table 3. General isolation and cultivation techniques.

Technique	Comment	References
Hungate technique	Anaerobic technique based on manipulation of materials under sterile, anoxic gas from probes; roll tube isolation	Hungate, 1969
Hungate technique	Thorough review or overview of Hungate technique	Bryant, 1972
Balch technique	Use of pressurized gas phases; culture transfers using anoxic sterile syringes; based on Hungate technique	Balch and Wolfe, 1976
Balch technique	Further development of Balch technique, including media, plate incubation, and larger-scale culture	Balch, et al., 1979
General anaerobic technique	Review with practical details	Hespell, 1990
	Also includes comments on larger fermentations and continuous culture	Ljungdahl and Wiegel, 1986
Techniques for clostridia	State of the art for 1969; some attention to medically important clostridia	Willis, 1969
Techniques for clostridia	General methods for the anaerobic sporeformers	Hippe et al., 1992
General technique	Enrichment, isolation and culture	Gottschal et al., 1992
General technique	General cultivation, with some emphasis on anaerobes	Tanner, 2002

oxygen than most clostridia. Indeed, the oxygen tolerance of clostridial species ranges from relative insensitivity, such as for *Clostridium aerotolerans* (Van Gylswyk and Van der Toorn, 1987) to extreme sensitivity, such as for some of the hydrogen-oxidizing acetogens (Drake, 1994).

The removal of oxygen and lowering the redox potential of culture media by the addition of a reducing agent are the two crucial parts of the technique. The removal of oxygen is usually achieved by boiling the medium. This will often be done with the medium under a stream of anoxic gas (Bryant, 1972) for a small batch of medium, or by steam sterilization in an autoclave for a large batch (above 1–2 liters). Steam sterilization is done with the vessel covered but still open to the atmosphere. After autoclaving, the medium is cooled under a stream of sterile, anoxic gas (Hungate, 1969), then sealed (Balch et al., 1979). Finally, sterile reducing agent is added to reduce the medium's redox potential. For preparing or dispensing anaerobic media in small batches such as in various tubes or serum bottles, the media are made anoxic by boiling, cooled to below 40°C, reduced by adding the appropriate amount of reducing agent, and then under the stream of oxygen-free gas, distributed into the tubes or bottles, which then are sealed with stoppers and aluminum crimps and autoclaved. In laboratories dealing with a lot of soil samples or various sporeforming strains, autoclaving time at 121°C should be extended to 30–40 min to ensure that heat resistant spores are killed (see the discussion of spore stability, especially of thermophilic clostridia and related species). In some instances, autoclaving the media a second time after incubating for 36–48 h at 30°C or 60°C is recommended for laboratories primarily working with thermophiles and thermobiotic soils and sediments.

A good grade of compressed gas, such as prepurified grade or better, is the starting point for provision of anoxic atmospheres. The gas is scrubbed free of oxygen in a heavy-walled copper tube (2.5×20–30 cm) packed with copper turnings and heated to 150–200°C in a tube furnace or with heating tape. Periodic exposure of this scrubber to hydrogen will readily regenerate the system. Sterile gas can be obtained by passing it through self-made gas filters, constructed from 2-ml glass syringe barrels with luer lock tips and packed with nonabsorbent cotton. Stainless steel needles, 17 gauge, complete the probe. These will tolerate many flame sterilizations, are small enough to be used with stoppered glassware without chipping it, but have a large enough bore for flushing syringes prior to making anoxic transfers.

A good anaerobic chamber equipped with an air lock greatly facilitates preparation of materials for anaerobic microbiology. The flexible chambers from Coy Laboratory Products, Inc. (Grass Lake, Michigan, United States) are economical and the easiest to use and maintain. The advantage of the anaerobic chamber is that it allows anaerobes and media to be handled as if they were on an open bench, facilitating plate transfers, dispensing of media, etc. Our chambers are operated with a gas phase of 1–2% hydrogen in nitrogen; oxygen levels of 2 parts per million (ppm) or lower (as measured by a palladium hydrogen probe) are easily achieved. The inclusion of activated charcoal gas scrubbers (to adsorb H_2S which poisons the catalyst) and an agent to remove moisture from the atmosphere (anhydrous calcium chloride pellets, 4–20 mesh) facilitate chamber and chamber catalyst (palladium on alumina pellets) maintenance. An anoxic solution of 1 N hydrochloric acid is used to clean and disinfect the anaerobic chamber.

This leaves no residue and its vapors are absorbed by the calcium chloride drying agent. Chambers seem to be easier to maintain if they are not used for culture incubations. The polyvinyl chloride (PVC) flexible bags of the chambers last more than 10 years, even with daily use. The use of ultraviolet (UV)-lamps for sterilization overnight or weekends is not recommended since it shortens drastically the life of the plastic bag.

A gas exchange station (Balch and Wolfe, 1976) allows control over the final atmosphere used for tube or plate culture of anaerobes. Elimination of as many joints and valves as possible, as well as construction from stainless steel components, simplifies the use and maintenance of a gas exchange station.

The use of a reducing agent to lower the redox potential of medium after the removal of oxygen is important for the culture of anaerobic bacteria. A variety of reducing agents are available, many based on sulfur chemistry (Hespell, 1990; Tanner, 1997; Tanner, 2002). Sodium sulfide solutions are commonly used. The authors use mainly cysteine-sulfide solution for most of their routine culture work. Cysteine (4 grams; free cysteine, not cysteine hydrochloride or another acid adduct) and anoxic water (boiled under an oxygen-free gas, then stoppered) are placed in an anaerobic chamber overnight. Sodium sulfide nonahydrate crystals (4 grams, freshly washed and dried) are taken into the chamber and dissolved with the cysteine in 100 ml of water. This is dispensed and stored in sealed tubes. For most media, 0.1–0.5 ml of this reducing agent is added per 100 ml of degassed medium taken into an anaerobic chamber for dispensing prior to sterilization. This reducing agent (prepared as just described) is stable for over a year.

General Medium Preparation

A general basal culture medium and the composition of stock solutions used to prepare it are given in Tables 4–7 (Tanner, 1997; Tanner, 2002). There are a number of other formulations of mineral, vitamin and metal stock solutions, many of which may be found in the *Handbook of Microbiological Media* by Atlas and Parks (1997). The desired carbon source, energy source, and electron acceptor, if any, are added to this basal medium for enrichment, isolation and culture of desired cell types. Attention to the type of microbial metabolism desired will indicate modification of this or any other basal medium recipe. For example, if a nitrogen-fixer were desired, ammonium salts would be eliminated from the medium. If strict control of any potential sulfate-reducing bacteria were required, chloride salts would be substituted for

Table 4. Basal medium.[a]

Component	Amount per liter
Mineral solution[b]	10 ml
Vitamin solution[c]	10 ml
Trace metal solution[d]	5 ml
Buffer[e]	2–10 g
Adjust pH as required	

[a]A general purpose medium adapted to pure culture or environmental studies upon the addition of the electron acceptor, electron donor and carbon source desired. A general source of growth factors, such as yeast extract (0.05–0.1 grams per liter), is often included.
[b]Described in Table 5.
[c]Described in Table 6.
[d]Described in Table 7.
[e]Good's buffers are recommended (Good et al., 1966). *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) should be used for neutral pHs, morpholinoethane sulfonic acid (MES) for slightly acidic pHs, and *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) for slightly alkaline pHs. Bicarbonate needed to balance carbon dioxide in the gas phase should be added after pH adjustment of the medium.

Table 5. Mineral solution.[a]

Component	Amount in grams per liter
NaCl	80
NH_4Cl	100
KCl	10
KH_2PO_4	10
$MgSO_4 \cdot 7H_2O$	20
$CaCl_2 \cdot 2H_2O$	4

[a]Adapted from Balch et al. (1979). Modified to reduce the number of required stock solutions. Includes calcium, which is often found in trace metal solutions.

Table 6. Vitamin solution.[a]

Component	Amount in mg per liter
Pyridoxine · HCl	10
Thiamine · HCl	5
Riboflavin	5
Calcium pantothenate	5
Thioctic acid	5
p-Aminobenzoic acid	5
Nicotinic acid	5
Vitamin B_{12}	5
MESA[b]	5
Biotin	2
Folic acid	2

[a]Adapted from Wolin et al. (1963). Includes a higher level of B_{12} compared to other vitamin solutions.
[b]Mercaptoethanesulfonic acid added for cultivation of the methanogenic archaea.

Table 7. Trace metal solution.[a]

Component	Amount in grams per liter
Nitrilotriacetic acid adjust pH to 6 with KOH	2.0
$MnSO_4 \cdot H_2O$	1.0
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.8
$CoCl_2 \cdot 6H_2O$	0.2
$ZnSO_4 \cdot 7H_2O$	0.2
$CuCl_2 \cdot 2H_2O$	0.02
$NiCl_2 \cdot 6H_2O$	0.02
$Na_2MoO_4 \cdot 2H_2O$	0.02
Na_2SeO_4	0.02
Na_2WO_4	0.02

[a]Adapted from Wolin et al. (1963). Modifications based on findings in the biochemistry of anaerobes. Other trace metal solutions often contain a source of boron and aluminum, as well.

any of the sulfate salts in the recipes. The growth of many anaerobes, even those that produce significant amounts of CO_2 as an end product, is stimulated by the presence of CO_2 in the gas phase. This CO_2 must be balanced by bicarbonate or carbonate to achieve the desired culture pH. In general, more bicarbonate is needed when the pH, incubation temperature, and partial pressure of CO_2 in the gas phase is higher. For example, to culture medium at pH 7.0 and in 20% CO_2 at a gauge pressure of 200 kPa, 2 and 4 grams of sodium bicarbonate per liter are added for incubation at 37°C and 60°C, respectively. For cultivation at pH of 6.0 or lower, bicarbonate additions can be omitted (this pH is outside of the bicarbonate/CO_2 buffer range). An advantage of using a gas phase that contains 10–30% CO_2 is that the amount of bicarbonate needed in the medium is small so that the bicarbonate can be sterilized as part of the whole medium. If a 100% CO_2 atmosphere is used, addition of bicarbonate or carbonate from a sterile stock solution to the medium is often necessary after steam sterilization to avoid precipitation or other problems. Bicarbonate is not stable above 50°C and forms CO_2 and carbonate. At concentrations above 1% the resulting CO_2 pressure can burst the culture vessels. Thus when elevated concentrations are needed, sodium carbonate can be used instead of bicarbonate. The pH is then adjusted by appropriate gassing with sterile CO_2 (i.e., passing the gas through cotton filled syringes).

Selection of Sporeforming Clostridia

As discussed above, not all members of the Clostridiaceae readily form spores. The sporeforming clostridia can be selected usually by treating with heat or alcohol (above 70% [v/v]), which kills or inhibits the growth of vegetative cells. Not all spores will survive these treatments and not all vegetative cells will be killed by these treatments, but this approach is still useful. The pasteurization procedure described in Hippe et al., (1992) is a very good example of the use of heat treatment to eliminate vegetative cells. The inoculum and a 0.9% saline blank are placed in an 80°C water bath. The blank has a thermometer to monitor temperature. The samples are held in the water bath for 10 min after 80°C is reached and then immediately cooled to room temperature. The specimens are now ready as inocula for enrichments or direct isolations.

For thermophilic species, the modified procedure of using 100°C for 1–2 min is recommended, which will heat-activate most spores from thermophiles. Some spores require a heat treatment of 100°C for complete activation (Byrer et al., 2000).

Treatment with ethanol is another good way to eliminate vegetative cells from an inoculum (Koransky et al., 1978). Suspended soil, sediment or liquids are mixed with an equal volume of sterile absolute ethanol and then incubated, preferably under anoxic conditions, for 60 min. Samples are now ready for inoculation into enrichments or for direct isolation procedures.

Enrichment and Isolation of Clostridia

In most cases, the numbers of the desired cell type are too small compared to the total number of bacteria for consistent or successful recovery. So, enrichments to increase these numbers need to be done prior to isolation. A combination of both approaches initially can be useful to recover desired clostridia, as often the different approaches will result in the isolation of different species. To illustrate this, consider the isolation of a clostridium that uses methanol as a carbon and energy source. Enrichments would be set up using a basal medium supplemented with a relatively low concentration of substrate (2 grams per liter) and with a relatively high concentration (10 grams per liter). One set of enrichments would receive a large inoculum and one set, a very small inoculum. This inoculum may be a suspension of 1 g of sediment in 10 ml of anoxic buffer. One ml of this suspension into 10 ml of enrichment medium would be a large inoculum and 1–2 drops, a small inoculum. One of the authors frequently uses inocula of 5–8 g (wet wt) or ml of sediment slurries per 100 ml of enrichment. Incubation at different temperatures could be another factor for this enrichment. Enrichment medium would be supplemented with 50 mg of yeast extract (Difco) per liter of medium. Simultaneously, direct isolation of

anaerobic methanol utilizers may be attempted in roll tubes (Hungate, 1969; Bryant, 1972) using medium with 1 g of yeast extract per liter. Again, a small and a large inoculum would be used. In all likelihood, methanol-fermenting bacteria may only appear in enrichments set up with the large inocula. However, the chances are that successful enrichments or isolations established under different initial conditions will yield isolates from different taxonomic groups.

The generalities of enrichment technique, some with emphasis on the clostridia, are summarized in many references, including Ljungdahl and Wiegel (1986), Hespell (1990), Gottschal et al. (1992), Hippe et al. (1992), and Tanner (1997) and Tanner (2002) (Table 3). The primary literature, such as the International Journal of Systematic and Evolutionary Microbiology (IJEM; formerly the International Journal of Systematic Bacteriology, IJSB) and Systematic and Applied Microbiology, should also be utilized routinely.

Some generalities are considered here: The basal medium given in Table 4 is a good starting point for many enrichments and culture media. Substrate is often added at 2–5 g per liter. The addition of yeast extract at 0.05–1 g per liter is often stimulatory, if not required (Adkins et al., 1992; Tanner, 2002). If desired, 5–20 mM electron acceptors (e.g., sulfate) are added. Hydrogen or carbon monoxide can serve as an electron source, and CO_2 as an electron acceptor. The presence of some CO_2 in the gas phase is often stimulatory, if not required. The inclusion of a buffer for pH control is usually recommended.

Undefined or commercially available media, such as cooked meat medium or trypticase soy agar, are generally not used for enrichments, unless one has treated a sample to eliminate vegetative cells and is recovering spores. They are more important for some isolations or standard culture of clostridia. It is not unusual to add significant amounts (2–10 g per liter) of yeast extract, trypticase or other complex nutrient sources for isolation and culture of clostridia.

Individual colonies of clostridia may be isolated from enrichments or directly from environmental samples by plating and incubation in anaerobic plate holders (e.g., Balch et al., 1979), bottle plates (Hermann et al., 1986), or roll tubes (Bryant, 1972). The authors have used all these techniques and have a slight preference for diluting out inocula in cooled, but still liquid (40–45ºC), agar and preparing roll tubes (termed "agar-shake roll tubes"). Subsequently, colonies can be picked for further liquid culture using a 1-ml syringe equipped with a slightly bent 3.8 cm, 20 gauge needle or using autoclaved Pasteur pipettes drawn out at the tip into a capillary and then bent. The colony is stabbed and the agar plug injected into a sealed serum tube

with an appropriate medium. Another isolation technique is to dilute an inoculum into cooled agar and incubate as solid butts. After colonies appear, these agar plugs can be aseptically removed from culture tubes, the colony aseptically exposed, using a syringe (or sterilized Pasteur pipette) and then transferred to a tube of liquid medium. Sealed serum tubes (e.g., no. 2048, Bellco Glass Co., Vineland, NJ, United States; Balch and Wolfe, 1976) or Hungate-type culture tubes (no. 2047, Bellco Glass Co.) are useful for routine culture. Cultures remain viable longer on agar surfaces than in broths especially when grown on mineral media in the presence of slowly metabolizable substrates. Agar slants can be prepared in sealed tubes and a drop of liquid inoculum injected through a stopper onto the top of the slant. This drop can flow down the slant, and growth on the agar surface will appear after incubation. There are a number of ways to prepare cultures of clostridia for long-term storage. One way is to obtain a good spore solution (i.e., a solution of highly refractile spores when examined by microscopy); spores of many species can be kept at 0–7°C for years without losing viability (J. Wiegel, unpublished data). A relatively easy method to maintain vegetative cultures that has been very successful in our hands is storage in glycerol (Wiegel, 1986; Tumbula et al., 1995). Anoxic glycerol (2 ml) is placed in sealed serum vials (10 ml) under a nitrogen gas phase and sterilized. A cell pellet is collected after gentle centrifugation (this can be done directly in sealed serum tubes with the appropriate adapters). The cell pellet is resuspended in 2 ml of either fresh or spent medium and injected into the vial. Stored at –20°C, the cells stay in liquid suspension, so little freeze damage occurs. Cells from as much as 20 ml of broth culture are readily concentrated for this 2 ml addition.

The culture of anaerobic clostridia is relatively easy after a little experience, almost as easy as that of *Escherichia coli* or a pseudomonad. Remember to make use of the general and primary literature as required and make use of experienced colleagues for training and advice.

Identification

Although nowadays most investigators identify their isolates using 16S rRNA sequence data, confirmation through comparison of the various properties of the different species is still important. As pointed out by several investigators, significant differences in morphology and physiology between isolates with very similar small subunit ribosomal RNA sequences (above 99% sequence similarity of the 16S rRNA gene)

can occur and lead to separation into different species.

Morphology

GRAM REACTION AND CELL WALL The phylogenetic branch of the Firmicutes is also called the branch of the "Gram positives" with low DNA mol% G + C, relating to the feature that the majority (see below) of the bacteria from this group stain Gram positive and exhibit a thick murein cell wall called "the Gram-positive cell wall type." Taking into account the above-discussed phylogenetic diversity, it is not surprising that the clostridial branch (Order Clostridiales) is very diverse and not homogenous in either overall morphology or biochemical characteristics of the cell components (Cato et al., 1986; Hippe et al., 1992; Dürre, 2004). On the basis of the 16S rRNA gene sequences, the Clostridiales contains both true Gram-type negative genera as well as those that only stain Gram negative. In fact, quite a large percentage of species among the Clostridiaceae (see species description) stain negative at all growth phases, regardless of the procedural modifications used (Popescu and Doyle, 1996; O'Brien and George, 1997). This includes species of *Clostridium* sensu stricto as well as "clostridia" species from genera no longer belonging to the family Clostridiaceae, e.g., *C. aceticum*, *C. arcticum*, *C. thermocellum* and *C. cellobioparum* (cluster III), *C. quercicolum* (cluster IX, now called "*Dendosporobacter*" within the Peptococcaceae), *C. formicaceticum* (cluster XI), *C. polysaccharolyticum* and *clostridiforme* (cluster XIVa). Thus, this discrepancy is widespread and not restricted to a specific cluster or subbranch of the Clostridiales. In addition, several species *C. tetani* (*Clostridium* sensu stricto) and *C. propionicum* (cluster XIVb) stain Gram negative in the mid to late exponential growth phase, whereas *C. indolus* and *C. puniceum* (*Clostridium* sensu stricto) stain Gram variable (*C. indolis* and *C. puniceum* [*Clostridium* sensu stricto]). To avoid confusion, i.e., including "Gram negative" species in the phylogenetic "Gram positive" branch, Wiegel suggested the term "Gram type" be used when referring to the systematic or phylogenetic position and "Gram staining" or "Gram reaction" when referring to the results of the Gram staining reaction (Wiegel, 1991; Wiegel et al., 1992). These terms will be used in the remainder of this chapter. The 16S rRNA gene sequence-based phylogenetic trees of the genera within the phylum Firmicutes defined as above clearly have to be labeled not only as "Gram-reaction negative" but also as "Gram-type negative," i.e., as genera of bacteria possessing a true Gram-type negative cell wall containing lipopolysaccharide (LPS). Prominent examples include a species from the family Heliobacteriaceae, the genus *Megasphaera* (non-sporulating) and, possibly, *Sporomusa*, the only Gram-type negative endospore former, although the presence of LPS has not been unequivocally demonstrated for the latter. Within the family of the Clostridiaceae, such examples have not been found, yet. Whether some Gram-type positive bacteria have a periplasmic or analogous space is still under discussion (Merchante et al., 1995; Navarre and Schneewind, 1999). The typical cell wall type for several clostridia, including the type species *C. butyricum* and all *Clostridium* sensu stricto species, is the *meso*-DAP direct type (*meso*-diaminopimelic acid is the direct bridging amino acid between the peptidoglycan strands). This type is also typical for the Gram-type negative cell wall. But most other members of the Clostridiaceae and Clostridiales, for which the cell wall has been determined, exhibit various cell wall types differing in the amino acids of the intervening peptide interbridges and side chains. These types are regarded as typical for Gram-type positive cell walls (for details, see Schleifer and Kandler, 1972), and various examples are found within the different clusters of Collins et al. (1994). Although in general, the specific cell wall type is regarded as a taxonomic feature for a genus, there are now exceptions to this. For example, the two alkalithermophiles *Clostridium paradoxum* and *C. thermoalkaliphilum* (cluster XI of Collins et al., 1994) are closely related with about 98% similarity in their 16S rRNA gene sequence. However, *C. paradoxum* contains the *meso*-DAP direct type, whereas *C. thermoalkaliphilum* has the L-ornithine-D-aspartate type (A4ß-type; Li et al., 1993; Li et al., 1994). Even closely related species (i.e., belonging to cluster XI of Collins et al., 1994) *C. formicaceticum* and *C. felsineum* contain *meso*-DAP, whereas *C. villosum* has ornithine-*iso*-D-asparagine, *C. glycolicum* contains no DAP but rather lysine and *iso*-D-asparagine, and *C. lituseburense* contains lysine and aspartic acid (Cato et al., 1986). Until the majority of the clostridial and related species have undergone cell wall analysis, the question of whether the cell wall type is a general taxonomic and systematic character for this group, remains open.

CELL SHAPES AND SIZES Given the heterogeneity of the Firmicutes, it is no surprise that a wide variety of morphological features are observed, although many species of the family Clostridiaceae exhibit typical rod-shaped cells. Many of the morphological descriptions of the long-known species are from Holdeman et al. (1977) and were updated by Moore et al. (1987) who described the anaerobic microorganisms when grown on peptone-yeast extract (PYG-media)

with or without added glucose (PY-media), or on blood or chopped meat agar. These media have become to some extent standard for describing mesophilic anaerobes. The morphology of some clostridial species cultivated on those different media change very little, while others change a lot in their diameter and length.

Most of the species belonging to the genus *Clostridium* sensu stricto and related genera are straight (e.g., *C. tetanomorphum* and *C. subterminale*) to slightly curved (e.g., *C. butyricum*) rods of different lengths and diameters, the ends of which vary from rounded and squared (e.g., *C. anthracis*) to tapered or pointed (e.g., *C. clostridioforme*, *C. perfringens* and *C. spiroforme*). During the exponential growth phase, the rod-shaped cells of most species are 0.5–1.8 μm but can be as thin as 0.3 μm (e.g., *C. sticklandii* and *C. barkeri*), as wide as 2 μm in diameter (*C. subterminale*), and 1.5–5 μm in length. Some species exhibit cells as long as 20 μm (e.g., when sporulating, see below) and even up to nearly 100 μm (e.g., *C. thermobutyricum*) under stress conditions when septation formation is affected. Nearly all Firmicutes species, especially anaerobes, exhibit pleomorphisms although to different degrees. These pleomorphisms include 1) irregular rods (e.g., *C. acetobutylicum*); 2) cells with bulging ends (observed frequently with many lactobacilli, clostridia and thermophilic anaerobes such as *Thermoanaerobacteria* when grown above their growth optimum temperature and under rich media conditions, e.g., the *Clostridium* sensu stricto species *C. palmarium* and *C. thermobutyricum*); 3) cells with true branching (e.g., *Thermobrachium* and *Anaerobranca* but so far not observed among *Clostridium* sensu stricto species); 4) cells forming long chains (e.g., *C. oroticum* → Lachnospiraceae; *C. bifermentans* → "Peptostreptococcaceae"); 5) cell aggregates forming twisted spaghetti-like structures at high pH or high temperature or both (*C. thermobutyricum*); 6) cells forming necklace-like chains with coccoid cells located alternatively between rod-shaped cells (e.g., *Thermoanaerobacter* species), which is due to nonsymmetrically dividing cells; and in connection with spore formation, 7) long filamentous cells (less than half the cell diameter, 3–10 times longer than the regular vegetative cells, e.g., *Clostridium thermocellum* (cluster III of Collins et al., 1994). But the family Clostridiaceae also includes species exhibiting mainly coccoid morphology (e.g., *C. coccoides* → Lachnospiraceae; *C. oceanicum* → *Clostridium* sensu stricto species); vibrio-like, nearly circular ring-shaped cells (e.g., *C. methylpentosum* → Lachnospiraceae; *C. cocleatum* → Collins et al. [1994] cluster XVIIIa; and spiral-shaped cells [e.g., *C. cocleatum* and *C. spiroforme*]).

One of the more obvious exceptions to the rod shaped morphology among the Clostridiaceae are the sarcina (anaerobic packet-forming bacteria). Sarcina-morphology is characterized by the uniquely shaped cell packets of of 4, 8, 12 or 16 and under some conditions larger clumps formed by divisions occurring more or less synchronously in 2 or 3 perpendicular planes, and the dividing cells are usually not separating into individual daughter cells. The "clostridial sarcina" (i.e., in the phylogenetic radius of *Clostridium* sensu stricto) are to be differentiated from the former sarcina, which are now classified in different taxa and some in different domains: the aerobic *Sporosarcina* (Bacillales), the packet-forming, asporogenic *Micrococcus*, the anaerobic *Methanosarcina* (domain Archaea), and the aerobic *Kineococcus* (Phillips et al., 2002). *Sarcina ventriculi* and related isolates are unique among Clostridiaceae because they produce extracellular cellulose fibers that aid in keeping the cells together (Canale-Parola et al., 1961; Canale-Parola, 1970).

COLONY MORPHOLOGY ON ANAEROBIC AGAR PLATES OR ROLL TUBES The colony form depends on whether the cells are grown on surface plates or on or within the agar of agar shake-roll tubes. Some species require a long adaptation and freshly prepared plates to be able to grow on or in agar-, Gelrite-, or Carrageen-solidified media because of their requirement of high water activity. Usually these isolates can be adapted by starting with soft agar and then transferring frequently to solid media containing increased concentrations of the solidifying agent. Many colonies located within the agar are of whitish to brownish color and disc shaped, and depending on gas production, split the agar. On plates, the colonies are round to irregular, raised or flat, translucent to white or brownish, shiny or dull. This morphology frequently depends on the type and age of the media.

FORMATION OF SLIME, CAPSULES AND SHEATHS Firmicutes do not form sheaths such as those formed by the extreme thermophilic Thermotogales or mesophilic leptothrix, nor is excessive slime formation (e.g., the formation of levan by some lactic acid bacteria) a property of species in the family Clostridiaceae. But similar to *Bacillus* and *Lactobacillus* species, several clostridia can form small capsules of various consistency and structure, which can be made visible with the traditional method of India ink using light microscopy. Most of these polysaccharide capsules are heteropolymers frequently containing uronic acids beside amino sugars. An unusual capsule is the one of *Sporosarcina* species (similar to *Bacillus* spe-

cies such as *B. subtilis*, *B. licheniformis* and *B. anthracis*) consisting of γ-polyglutamic acid or a γ-D-glutamyl polymer (Troy, 1973; Kandler et al., 1983). Furthermore, many clostridia, produce slime material when growing in biofilms and on surfaces, e.g., in fermentors during continuous culture of, e.g., *C. thermobutyricum* (Canganella and Wiegel, 1999) or *Morella thermacetica* and *M. thermautotrophica* (Wiegel et al., 1991).

FLAGELLA, PILI AND FILAMENTS Many Firmicutes are peritrichously flagellated. However, in many instances the number of flagella is low (i.e., 3–10). Pili have also been described (e.g., in *Moorella glycerini*; Slobodkin et al., 1997), but for most described species, the occurrence has not been investigated carefully by electron microscopy so it is not clear whether pili occur more rarely in clostridia than in, e.g., proteobacteria or lactic acid bacteria.

An unusual morphological feature is observed with clostridia isolated from acid forest soil. *Clostridium acidisoli*, *C. akagii* (both *Clostridium* sensu stricto species), and *C. uliginosum* form chains or aggregates linked by connecting filaments (each composed of a core and outer sheath) that have no known function (Kühner et al., 2000; Matthies et al., 2001).

STRUCTURES ASSOCIATED WITH EXTRACELLULAR PROTEIN (COMPLEXES) Members of the Clostridiales exhibit several different methods of excreting and attaching various surface proteins to the outer cell wall envelope (for details, see Navarre and Schneewind, 1999).

S-layer The most recognizable among the surface layer proteins are the S-layer proteins, all of which are widespread among the Firmicutes. These self-assembling subunits occur usually in typical geometric arrays on the surface, as observed by electron microscopy. Most arrangements are hexagonal, tetragonal and oblique (Sara and Sleytr, 2000). The pattern is independent of the bacterial cell on which they assemble. Although the pattern of the S-layer proteins is species–specific, they are presently not regarded as genera-specific among the Firmicutes. Several clostridia and related species contain a nongeometrically structured protein layer of enzymes, without any visible regular geometric pattern of proteins or protein-carbohydrate complexes. Some clostridial and related species exhibit extracellular enzymes with S-layer-like sequences and are attached to structures on the outside of the cell wall (Sara, 2001). S-layer sheets have been suggested for use as matrix material to support enzymes (Sleytr et al., 1996).

Structures Associated with Extracellular Protein (Complexes) Some enzymes form special protein complexes or structures on the outer cell envelope (Navarre and Schneewind, 1999). A special configuration of extracellular enzymes has been found in the anaerobic, thermophilic and mesophilic clostridia and related bacteria which are able to hydrolyze crystalline cellulose. The cellulolytic enzymes are excreted as large (4–8 MDa) enzyme complexes called "cellulosomes" (Bayer and Lamed, 1986) containing various cellulases, xylanases and esterases (e.g., feruloylesterase, which cleaves the cellulosic strands from lignin [Bayer et al., 1994; Doi et al., 2003; Madkour and Mayer, 2003] and forms polycellusomes [Mayer et al., 1987]). Correspondingly, xylosomes associated with xylanolytic anaerobes have been proposed but not yet unequivocally demonstrated for any low G + C Gram-positive strains. However, for several of the anaerobic Firmicutes (e.g., *Thermoanaerobacter* and *Thermoanaerobacterium*), protrusions of cell wall components have been observed to contain extracellular starch hydrolyzing enzymes (Antranikian et al., 1987).

SPORULATION (FIG. 2) One of the most characteristic properties of subgroups within the low G + C Gram-positive phylum is the formation of endospores. Some members of the high G + C Gram-positive group, for example those in the class Actinobacteria, form conidiospores, which differ sharply from endospores both in structure and formation. However, other genera of the Firmicutes, such as *Corynebacteria*, *Bifidobacterium*, *Athrobacter*, *Gardnerella*, and related bacteria are asporogenic (i.e., form neither conidospores nor endospores).

The extent and conditions for endospore formation varies greatly among the genera, species, and even between clones of the same strain in different laboratories (e.g., as demonstrated for *Moorella thermoacetica*; Wiegel et al., 1991). The extent of sporulation ranges from nearly 100% sporulation at end of the exponential growth phase in the presence of utilizable substrates (as in *C. paradoxum* → Lachnospiraceae; Li et al., 1993) to less than 0.01% sporulation under various starvation and sporulation-favoring conditions (as in *Thermoanaerbacter brockii*; Cook et al., 1991). However, although many species and strains among the Firmicutes do not exhibit spore formation under any of the standard sporulation conditions (e.g., stationary phase, starving conditions, unfavorable growth parameters, and growth on chopped meat agar), most possess most (if not all) of the sporulation-specific genes (Brill and Wiegel, 1997; Stragier, 2001; R. Onyenwoke and J. Wiegel, unpublished data). Since more than 75 gene products are
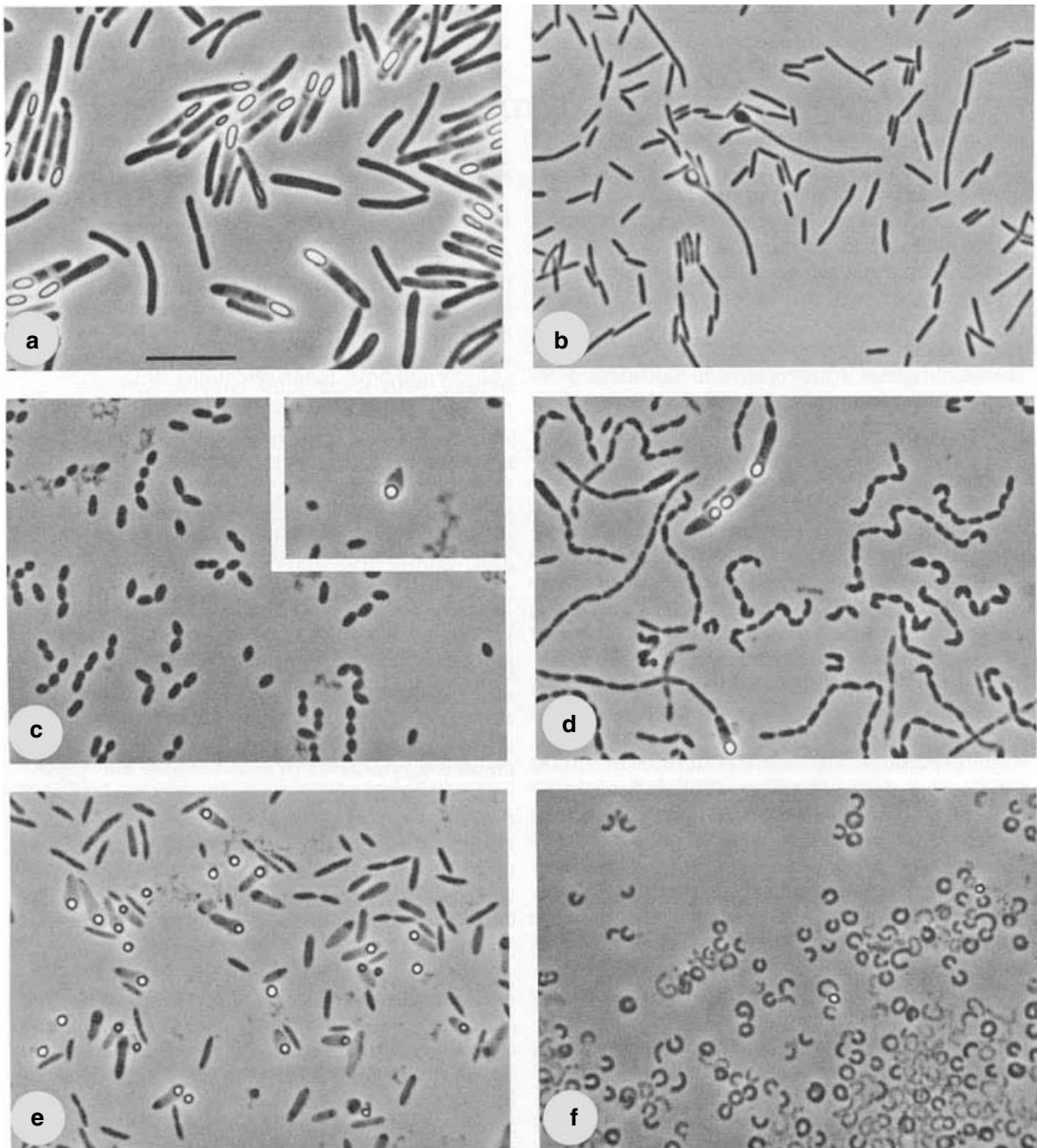
Fig. 2. Phase-contrast photomicrographs of some representatives of the genus *Clostridium*. Bar = 10 μm. (a) *C. butyricum* DSM 552; yeast extract-peptone agar; large, straight or slightly curved rods with round ends; sporangial cells slightly distended; oval, subterminal spores. (b) *C. thermocellum* DSM 1313; cellobiose medium; showing elongated sporulating cells with oval terminal spores. (c) *C. coccoides* DSM 935; CMC medium; coccobacilli to rod-shaped cells forming pairs and chains; round subterminal spores. (d) *C. oroticum* DSM 1287; CMC agar; rods occurring in long, tangled chains; enlarged sporangial cells with oval terminal-to-subterminal spores. (e) *C. indolis* DSM755; CMC agar; with round terminal-to-subterminal spores. (f) *C. cocleatum* DSM 1551; CMC agar; showing preponderance of semicircular-to-circular cell forms; oval spores are subterminal to terminal. (g) *C. bifermentans* DSM631; CMC agar; central-to-subterminal oval spores not distending cells. (h) *C. sporogenes* DSM 767; peptone-yeast extract-glucose medium; oval subterminal spores swelling the cells slightly. (i) *C. acetobutylicum* DSM 792; milk agar; rods of varying length; sporeforming cells cigar-shaped; spores oval to cylindrical, subterminal. (j) *C. ramosum* DSM 1402; CMC agar; showing tendency to Y- and V-shaped cells. (k) *C. cadaveris* DSM 1284; CMC agar; rods with oval terminal spores. (l) *C. tetanomorphum* DSM 665; CMC agar; large, straight to slightly curved rods; nearly round terminal spores. (From The Genus Clostridium—Nonmedical in the second edition.)
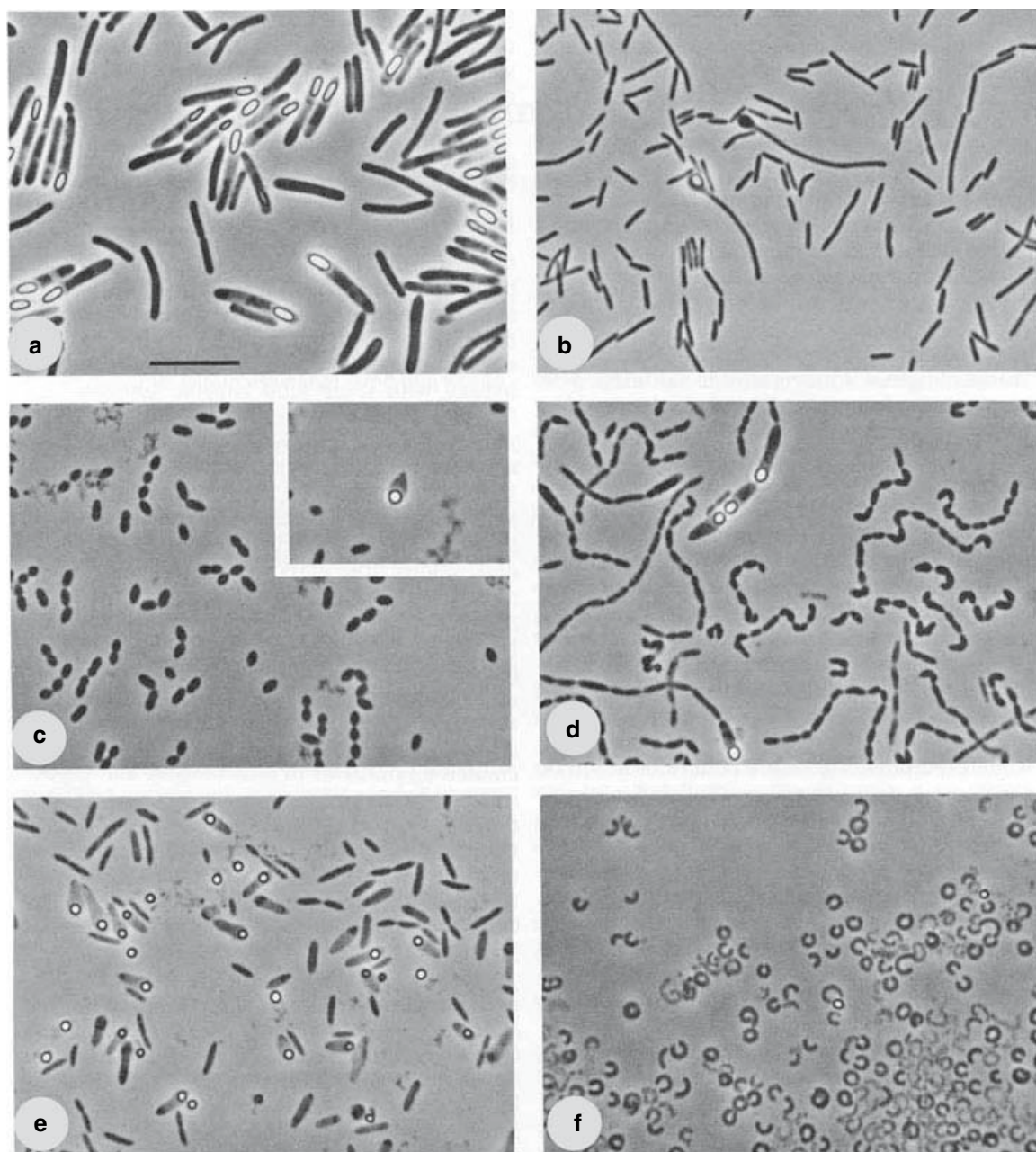
Fig. 2. *Continued*

sequentially required for sporulation, it is obvi-
ous that a mutation rendering one of the
required gene products nonfunctional leads
to naturally occurring sporulation-deficient
mutants and strains. This is especially true for
bacteria that grow like thermophiles in geother-
mally heated environments but under similar
continuous culture conditions. The isolation of
strains that lack observable sporulation is com-
mon among, e.g., butanol- and acetone-forming
C. acetobutylicum (Meinecke et al., 1984). The
reversion of sporulation-negative mutants (e.g.,

of *Bacillus pumilis*) to sporulating phenotypes
via bacteriophage treatment has been demon-
strated (Bramucci et al., 1977). Consequently,
species and strains that contain sporulation
genes but do not exhibit heat-resistant spore for-
mation are usually now termed "asporogenic" in
contrast to nonsporulating species that do not
possess most of the sporulation specific genes
(Brill and Wiegel, 1997; Byer et al., 2000). How-
ever, among the Firmicutes are genera assigned
to this branch on the basis of 16 rRNA gene
sequence analysis (see above), for which no

spore formation has been observed. Yet based on *B. subtilis* genome analysis and genome sequence comparison, some sporulation specific genes have been found (in Gram-type negative *Megasphaera elsdenii* or the Gram-type positive *Streptococcus*, *Staphylococcus*, *Listeria* and *Enterococcus*). The previous (Hippe et al., 1992) requirement of spore formation for the genus *Clostridium* sensu stricto or related genera is thus no longer valid. Another example is that absence or presence of spores cannot be used to differentiate between *Tissierella praeacuta* and *C. hastiforme* (Peptostreptoccocaeae) because *Tissierella* contains specific sporulation genes (R. Onyenwoke et al., unpublished results). Thus, the *T. praeacuta* type strain is regarded now as asporogenic with *C. hastiforme* as a sporulating strain.

The test for spores involves use of phase contrast light microscopy and heat resistance. To induce sporulation many *Clostridium* and related species and genera are grown on agar, especially chopped meat agar, or with poorly utilized sugars, which apparently does not lead to extremely heat resistant spores. Under the light microscope with phase contrast, forespores are black, whereas mature spores appear as bright refractile objects either still inside the more dark intact cytoplasm or less dark mother spore, or appear as spores released into the medium that may be associated with cell debris or cell material. Released spores (e.g., of *C. paradoxum*) can be clumped together in a gelatinous matrix (like frog eggs). The heat resistance test determines culture viability after a heat treatment at 80°C (mesophiles) to 100°C (thermophiles) for 10–20 min. Whereas most spores of mesophiles have a D-10 (exponential killing) time of less than a min at 121°C, spores of thermophiles (e.g., *Moorella thermacetica* strains) can have a D-10 time of up to 2 h (or above 1 h for *Thermoanaerobacterium thermosaccharolyticum*) at 121°C. The thermostability and drying resistance of spores can vary drastically with growth conditions (e.g., heterotrophic versus autotrophic) or temperature during sporulation (Byrer et al., 2000). So far, for *Clostridium* sensu stricto species, such high thermoresistance (especially of spores of pathogenic species) has not been observed, although note that for several species this feature has not been extensively tested.

Most of the species form only one spore either round or oval which is terminally, subterminally or centrally located in the mother cell, either without distending the mother cell (frequently seen for spores of *Bacillus* species with central locations but also seen in e.g., *C. bifermentans* and the type species of the genus *Clostridium*, *C. butyricum*, exhibiting central to subterminal oval

spores) or causing swelling of the mother cell (as seen, e.g., in *C. clostridioforme*, *C. chauvoei* and *C. sordellii*, which have central oval spores). In clostridial species (more rare in *Bacillus* species), round to slightly oval spores are frequently terminally located and distend the cells up to two times the width of exponentially growing vegetative cells and thus are often referred to as the "drumstick sporulation type." In several of these species, sporulation is accompanied by the formation of cells that are 2–10 times the length of a vegetative cell and either slightly less or much less than half the vegetative cell diameter (e.g., *C. hastiforme*, *C. kluyveri*, *C. lituseburense* and *C. sartogoforme*). The elongation occurs usually at the onset of the sporulation before forespores are visible (e.g., as in *C. thermocellum*). However, note that there is no real relation between specific sporulation type and specific group or genus (e.g., *Clostridium* = drumstick type versus *Bacillus* = central or subterminal type without a swollen sporangium, etc.) as has been depicted frequently in textbooks.

A few species among the Clostridiaceae produce two or more endospores. Examples include the bisporic *C. disporicum*, *C. oceanicum*, *C. lentocellum* and the polysporic *Anaerobacter polyendosporus*, which is closely related to *C. intestinale* (Murray et al., 1987; Siunov et al., 1999). One of the most interesting sporeformers is "*Metabacterium polyspora*," a symbiont of guinea pig gastrointestinal tract and the various fish symbionts "*Epulopiscium*" sp. (→ Lachnospiraceae). "*Metabacterium*" besides exhibiting symmetric cell division propagates mainly through forming several endospores by asymmetric division at both poles, which normally would only lead to two subterminal spores. However, the engulfed cell (forespore) of "*Metabacterium*" starts to divide by symmetric division leading to 2–9 spores per cell (see Angert and Losick [1998] and literature cited therein). Here sporulation is apparently converted from just being a dormancy and survival mechanism for overcoming temporary adverse environmental conditions to an unusual way of propagation, well-suited to the lifecycle of this intestinal symbiont. It is suggested that this could be the intermediate form between clostridial or bacillus-type sporulation and the viviparous formation of multiple offspring by internal reproduction in "*Epulopiscium*" (→ Lachnospiraceae; Collins et al. [1994] cluster XIVb) and related members of the order Clostridiales. So far, such features have not been observed within the Clostridiaceae. Since the intestinal flora of most insects (and other invertebrates) and of vertebrates are not known in detail, the existence of more bacteria belonging to the Firmicutes branch and even to the family Clostridiaceae, with this or similar, pres-

ently unusual, sporulation behavior can be expected (Angert and Losick, 1998).

PIGMENTS Unlike many aerobic and airborne bacteria, most cells of this group as well as most anaerobes do not contain bright pigments such as carotinoid derivatives. Surface colonies can be translucent to dull white and buff-colored to dark, dull or shiny gray. Cell pastes are frequently greenish gray or grayish black because of metal sulfide formation, although when grown under specific conditions can be brown to yellow brown because significant amounts of electron carriers such as ferredoxins and flavins are present. One unusual case is *C. puniceum*, belonging to *Clostridium* sensu stricto, which is pink when grown on potato-infusion agar but whitish when grown on blood or complex media (Lund et al., 1981).

Physiology

DIVERSITY The physiology and metabolism of members of the Clostridiaceae varies greatly and utilizes many different metabolic principles. The Clostridiaceae includes psychrophilic to thermophilic species (no hyperthermophiles have been reported within the Firmicutes), moderate halophiles, moderately acidotolerant species (up to pH 4.2), and alkaliphiles (pH$^{25C}_{opt}$ at or above pH 9). With respect to carbon source utilization, species include chemolithoautotrophs and various organoheterotrophs. The latter vary widely and include 1) saccharolytic species (which are able to use many or a few monocarbohydrates and one or more polymers such as starch, chitin, xylans and cellulose [but not lignin; lignin degradation under anaerobic conditions has not been demonstrated and is presently regarded as unlikely because lignin depolymerization proceeds via a radical mechanism]); 2) proteolytic and peptidolytic species and pairs of amino acid utilizers; 3) lipolytic and purinolytic species; 4) organic acid utilizers; and 5) C-1 compound utilizers (heterotrophic methylotrophs and chemolithoautotrophic C-1-utilizers).

Although most syntrophic Firmicutes species are now placed in the separate family Syntrophomonadaceae (Clostridiales), there is still one syntrophic *Clostridium* species, *C. ultunense*.

PHYSIOCHEMICAL BOUNDARIES FOR GROWTH *Anaerobic Metabolism and Oxygen Sensitivity* Generally clostridial metabolism is synonymous with obligately anaerobic metabolism, i.e., growth is not based on ATP formation using electron transport chain phosphorylation with O_2 as terminal electron acceptor (Gottschalk, 1986). None of the described *Clostridium* sensu stricto species (unlike *Desulfovibrio*; Escheman et al., 1999) has been shown capable of making ATP using O_2 as an electron acceptor. But even this characteristic, viewed in the past as the most fundamental one for the definition of clostridia and members of the Clostridiaceae, might not be true anymore. So far, no member of the family Clostridiaceae is truly facultative or obligately aerobic, using the above definition. But 16S rRNA gene sequence based phylogeny places the *Thermaerobacter* species (which are obligately aerobic thermophilic and sporeforming [except for the type species]) into cluster VIII of the Syntrophomonadaceae, despite the high G + C mol% of 72.5 for the type species (Takai et al., 1999; Nunoura et al., 2002; Spanovello et al., 2002).

Although they cannot use O_2 as terminal electron acceptor, many members of the Clostridiaceae can tolerate O_2, especially under nongrowth conditions, i.e., in the absence of utilizable substrates and energy sources. Many clostridial species stop growing when exposed to O_2 but resume growth under reinstated anoxic conditions (e.g., *C. butyricum*, *C. acetobutylicum* and *C. intestinale* [*Clostridium* sensu stricto], *C. aerotolerans* → Lachnospiraceae [O'Brien and Morris, 1971]; *C. paradoxum* → Peptostreptococcaceae, J. Wiegel, unpublished data). The oxygen concentration tolerated depends on the genus and species. Some species can grow under microaerophilic conditions, such as *C. haemolyticum* (*Clostridium* sensu stricto), which grows below a pO$_2$ of 0.5% (v/v), and *C. novyi* type A, which grows in the presence of up to 3% O_2. On the other hand, *C. novyi* type B (*Clostridium* sensu stricto) is killed when exposed to oxygen for a few min. When grown in nonagitated liquid cultures exposed to air, some species (e.g., *C. aerotolerans* → Lachnospiraceae; Van Gylswyk and Van Toorn, 1987) can reduce the redox potential of their medium below –120 mV (using resazurin as indicator). Cytochromes, menaquinones, catalase and superoxide dismutase are present at various levels. However, presently it is not known how strong the correlation is between the concentrations of the electron acceptors and enzymes and the O_2-tolerance of a specific strain. In many species the concentrations are below detection limits. In others they are at relatively high concentrations and can vary more than 60-fold depending on growth and stress conditions (McCord et al., 1971; Gottwald et al., 1975; Ashley and Shoesmith, 1977; Gregory et al., 1978; Cardenas, 1989) The oxygen sensitivity can be for various reasons, including 1) the usual damage of sensitive cell components by peroxides and radicals formed by interaction of media components and enzymatic reactions with O_2 and 2) specific effects such as the inhibition of the reduced nicotinamide adenine

dinucleotide (NADH)-oxidase causing a severe shortage of NADH (Uesugi and Yajima, 1978). In *C. aminovalericum*, a highly active NADH-oxidase with a K_m of ca. 62 μM O_2 is responsible for keeping the liquid media practically anoxic, thus this bacterium can grow in the presence of 3% O_2 (Kawasaki et al., 2004).

Temperature Most of the presently known members of the Clostridiaceae are mesophiles growing well in the range from around 10°C to slightly above 40°C. Two closely related moderate thermophiles, *C. palmarium* and *C. thermobutyricum*, with maximal growth temperatures below 65°C belong to the *Clostridium* sensu stricto. Most (extreme) thermophiles have been moved to different families (e.g., the well-studied *C. thermohydrosulfuricum* and *C. thermosaccharolyticum*, now species of the Thermobacteriaceae, *Thermoanaerobacter* and *Thermoanaerobacterium*, respectively). Several other thermophiles and extreme thermophiles (T_{max} >70°C to <80°C) still belong to the Clostridiaceae and are grouped together in the genus *Caloramator* (type species is the former *C. fervidus*) with species having the highest T_{max} values of around 75°C among the Clostridiaceae, closely related *Thermobrachium celer* and *Thermohalobacter berrensis*.

On the other end of the temperature spectrum are quite a few psychrotolerant and psychrophilic (T_{opt} below 15°C) Clostridiaceae species, including *C. arcticum*, *C. vincetii*, *C. algidicarnis* and *C. algidixylanolyticum*). Several psychrophilic and tolerant species were isolated recently from vacuum-packed meat (e.g., *C. estertheticum*, *C. frididicarnis*, *C. gasigenes* and *C. laramiensse*). One can assume that in the near future more psychrotolerant and psychrophilic clostridial species will be isolated owing to the renewed interest in low temperature-active enzymes for biotechnology (e.g., Cato et al., 1986; Broda et al., 2000).

pH Many clostridial species are neutrophiles although they can lower the pH to below 4.8 after 4–5 days of incubation in complex media. A few species such as the *Clostridium* sensu stricto species *C. acidisoli* and *C. akagii* (along with *C. pasteurianum* its closest relative; Kühner et al., 2000) are regarded as acid tolerant and able to grow atpH 3.6–7.0 with no distinct pH optimum, and at pH 4.0–9.0 with an optimum around 6.5 (Matthies et al., 2001). These isolates (with doubling times as short as 3.5 h at pH 4.0) are from acid forest soil. Most species cannot initiate growth at such low pH values and some species cannot grow below pH 6.2. However, cells may continue to metabolize and lower the pH further while their extracellular enzymes

continue to function (e.g., the cellulase complex of *Clostridium thermocellum* continues to degrade cellulose in mixed cultures down to pH 4.0). Some strains show only a slight or no acidification, and pH values in the cultures stay above pH 6.2 such as cultures of solvent-producing glycolytics or proteolytic, peptidolytic, and ammonium-producing strains growing in rich media (e.g., *C. sticklandii* and *C. aminophilum*). In butanol-producing species, a pH below 5.0 caused by the formation of butyric and other acids will trigger these bacteria (i.e., *C. acetobutylicum* and *C. beijerinckii*) to switch from acid production to the formation of pH-neutral compounds such as butanol and acetone, or butanol and ethanol, and even re-utilize the produced acids.

The inability to grow at low pH is used for excluding clostridia from fermentation processes to prevent production of, e.g., butyric acid during the lactic acid fermentation of silage or sauerkraut. Clostridial products such as sulfide or butyric acid would spoil the taste and smell, thus farmers acidify silage or cabbage by adding acetic acid or even mineral acids at the start of the fermentation. On the alkaline side, several clostridia can grow at pH values above 8.0, and even well above 8.5 (e.g., *C. histolyticum*) although their optimum is at neutral pH values (alkalitolerant species). True alkaliphilic species are the thermophilic *C. paradoxum* and *C. thermoalkaliphilum* ($\rightarrow$ Peptostreptococcaceae) with pH$^{25C\ optima}$ around pH 10. Souza (1974) reported on the isolation of alkaliphilic (growth up to pH 11.2) spore-forming anaerobes, but to date no species have been validly published. *Natronincola* (family Clostridiaceae; Zhilina et al., 1998) stands out among the true alkaliphiles, since it is also a moderately halophilic mesophile able to grow at pH values up to 10.5 with an optimum around 9.5.

Growth Rates Typically the shortest observed doubling times under optimal growth conditions for saccharolytic growth on monosaccharides is between 30 min and 3 h but can be as short as 10 min (e.g., in *Thermobrachium celer*, the fastest growing member of the Clostridiaceae; Engle et al., 1996). When growing on substrates such as crystalline cellulose (*C. thermocellum*; Freier et al., 1988) or chemolithoautotrophically, the shortest observed doubling times are about 5–8 h.

METABOLIC DIVERSITY The clostridia harbor quite a lot of physiological specialists, although the reassignment of many former *Clostridium* species has somewhat narrowed the spectrum. Many clostridial and related species require yeast extract or similarly complex media compo-

nents for optimal growth. The family Clostridiaceae includes species with broad substrate spectra but also species with narrow substrates. Some chemolithoautotrophic species (e.g., the iron(III)-reducing *Clostridium* species closely related [above 99% similarity at the 16S rRNA gene sequence level] to *C. thermobutyricum*; Wiegel et al., 2003) generate their ATP only via electron transport phosphorylation. With respect to fermentation products, family Clostridiaceae members include pH-neutral solvent producers, mixed acid and alcohol producers, and homoacidogenic fermenters.

Many of the *Clostridium* sensu stricto species exhibit mixed acid and alcohol fermentations, meaning they also form, besides the "genus-specific" product butyric acid, varying concentrations of acetic acid, lactic acid and/or ethanol, propanol or butanol. Similar to many other clostridial species, species which produce butyrate while growing on sugars use the Emden-Meyerhof-Parnass (glycolysis) pathway to convert sugars to pyruvate and then use pyruvate-ferredoxin oxidoreductase, acetyl CoA acetyltransferase, and some pathway-specific enzymes (for pathways, see Gottschalk [1986], White [1995], and Lengler et al. [1999]). The amount of butyrate produced depends strongly on the growth conditions. The *Clostridium* sensu stricto harbors the less-studied mesophile *Clostridium ljungdahlii*, which exhibits homoacetogenic fermentation that involves use of the Ljungdahl-Wood pathway and the characteristic enzyme CO-dehydrogenase/acetyl-CoA synthase found as well in the well-studied thermophile (now identified as *Moorella* species in the Thermoanaerobacteriaceae), which also exhibits homoacetogenic fermentation (Collins et al., 1994). *Clostridium formicoaceticum*, physiologically a very similar mesophile, belongs to cluster XI (Collins et al., 1994). The alkaliphilic, moderate halophilic *Natrononicola histidinovorans* uses the Ljungdahl-Wood pathway (acetyl-CoA pathway), but instead of using sugars as substrates, it oxidizes only histidine, glutamate and pyruvate (Zhilina et al., 1998). Despite its fermentative metabolism, *N. histidinovorans* proved to be obligately dependent on the activity of ion pumps (Pusheva et al., 1999). Lactate is a frequently observed fermentation product, especially with the saccharolytic species. Although several clostridia are inhibited by lactate concentrations far below 40 mM, some species can produce high concentrations (more than 150 mM) of lactate as the main fermentation product (*C. stercorarium* subsp. *thermolacticum*). Lactate, however, can serve as substrate for other fermentation specialists including *C. homopropionicum* (*Clostridium* sensu stricto), *C. propionicum*, *C. neopropionicum* and *C. lactati-*

fermentans (all cluster XIVb; Collins et al., 1994) exhibiting fermentation of lactate to propionic acid and acetate using the acrylate pathway. They can also utilize other substrates such as ethanol and CO (in the case of *C. neoproprionicum*) and amino acids via pyruvate disproportionation (in the case of *C. propionicum*). This acrylate pathway is also used by other members in different families of the Clostridiales, e.g., *Megasphaera* (Seeliger et al., 2002). The energetically more efficient succinate pathway to form propionate used by the propionibacteria is not used by members of the Clostridiaceae (Dorner and Schink, 1990; Tholozan et al., 1995; Van der Wielen et al., 2002).

Clostridium sensu stricto species (e.g., *C. beijerinckia* and *C. acetobutylicum*) produce another well-known clostridial fermentation product butanol together with either ethanol or acetone. This fermentation uses initially the same pathway as the butyric acid fermentation and reduces the acid moiety to the pH neutral compound butanol (using the acid as an additional electron acceptor). Many *C. butyricum* strains produce as a main alcohol propanediol when growing on glycerol. No longer among the Clostridiaceae do the species exhibit a yeast-like ethanol fermentation. These species have now been moved to Thermoanaerobacteriaceae. However, some *Clostridium* sensu stricto species can produce relatively high concentrations of ethanol from peptides and proteins, a property that can lead to elevated post-mortem blood ethanol concentrations (Wiegel, 1980).

A special physiological case is represented by *C. kluyveri* which using a special pathway involving acyl~CoA transferases is able to use ethanol and reduce it to caproate and butyrate with acetate as the oxidized acid product. This species can also use crotonate as substrate for growth, producing butyrate, 2 acetates and 1 ATP. This latter pathway is also used by obligate syntrophic clostridia species belonging now to the family Syntrophomonadaceae.

An environmentally and physiologically interesting group is the purinolytic clostridia (e.g., *C. acidurici* and *C. purinolyticum*), which use purines, uric acid derivatives, and pyrimidines. They use special pathways with their interesting selenocysteine-containing enzymes (e.g., purine hydroxylase and xanthine dehydrogenase; Dürre et al., 1981; Self, 2002) to anaerobically degrade nucleic acids and their hydrolysis products. Somewhat related are other heterocyclic compound degraders that degrade nicotinic acid and pyridine derivatives (Kaiser et al., 1996; Andreesen, 2003). Many of these also can utilize amino acids. A great variety of degradation pathways for amino acid utilization exist among the Clostridiales and even among the Clostridiaceae

depending on the amino acid and clostridial species. These pathways frequently include the hydroxyacid pathway, the methylaspartate pathway for rearranging the carbon skeleton, and B12-dependent enzyme reactions. For an overview of the different pathways, which is outside of the scope of this chapter, see The Anaerobic Way of Life in volume 2. Among the special amino acid-utilizing clostridial species worth mentioning here are *C. sticklandii* (→ Peptostreptocaccaceae) and related species. These perform the Stickland reaction with amino acid pairs: one is oxidized to an acid that is one carbon shorter, and the other is reduced to the corresponding fatty acid. Environmentally important species also include clostridia (e.g., *C. methoxybenzovorans* (→ Lachnospiraceae), and the *Clostridium* sensu stricto species *C. pfennigii* (forming butyrate) and *C. scatologenes*) that can use methoxy aromatic compounds. This reaction is regarded as important in the anaerobic degradation and mineralization of lignin and because of its breakdown products including vanillic acid.

Genetics

Knowledge of the genetics of clostridia and how to manipulate DNA in several clostridial species has greatly expanded in recent years. Most of the work in the area has focused either on the pathogenic clostridia or the (potentially) important solvent-producing clostridia. However, the basic information should apply to many other species in the Clostridiaceae.

Fortunately, much of the knowledge on the genetics of clostridia has been summarized in a number of review articles over the past 15–20 years. A review by Rogers (1986) summarized the literature on mutation of clostridia, plasmids in clostridia, genetic transfer, and cloning of genes in *E. coli*. Ultraviolet (UV) light, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, and the combination of UV and especially ethylmethanesulfonate has been used to obtain clostridial mutants. Minimal defined media for *C. acetobutylicum* were identified (e.g., Long et al., 1983), making the isolation of mutants with auxotrophic markers feasible. Plasmids, primarily cryptic, were isolated from eight clostridia, primarily pathogenic species or solvent-producing species. Clostridial bacteriophages are known, and the literature on these has been reviewed (Mahony, 1979). Conjugation in *C. perfringens* has been demonstrated (Abraham et al., 1985), as has transformation using protoplasts.

By the time a review by Young et al. (1989) was published, the more promising technique of electroporation had been used as a tool for transformation in clostridia (Allen and Blaschek,

1988). Transposons, which can be used for mutagenesis or cloning, were available for clostridial systems (Wust and Hardegger, 1983). A number of clostridial genes had been cloned into *E. coli* (Youngleson et al., 1988). This is a useful research tool, but direct genetic manipulations in a clostridial species itself will be required for improving applications in solvent production.

Several chapters covering bacteriophages, plasmids, transposon mutagenesis, and transformation in pathogenic clostridia are presented in a book edited by Rood et al. (1997). It contains a chapter on the phylogenetic heterogeneity, or complexity, of the clostridia (Stackebrandt and Rainey, 1997), along with a good review of sporulation in clostridia (Labbe and Shih, 1997).

A chapter on the genetics of clostridia by Mauchline et al. (2000) appeared in the second edition of the *Manual of Industrial Microbiology and Biotechnology* (Demain and Davies, 2000). The genetics of solvent-producing clostridia was the focus of this chapter. Procedures reviewed included transformation by electroporation, use of broad-host-range conjugative plasmids for genetic manipulation, and use of conjugative transposons for genetic manipulations.

A recent review by Tummala et al. (2001) on genetic tools for clostridia also focused on the solvent-producing clostridia. Two other procedures for manipulating these clostridia were described, including development of gene expression reporter systems (Tummala et al., 1999) and the use of antisense RNA for metabolic engineering of clostridia (Desai and Papoutsakis, 1999).

Mai and Wiegel published an overview of the genetics of thermophiles including thermophilic Firmicutes (Mai and Wiegel, 1999; Mai and Wiegel, 2000), which should also apply to the thermophiles of the clostridia sensu stricto. Spheroplasts (autoplasts) from *C. thermobutyricum* have been regenerated (J. Wiegel, unpublished data) by the described methods used for other Firmicutes.

An important, and still not fully exploited, advance in the genetics of clostridia is complete genome sequencing. Genome sequences are now available for *C. perfringens*, *C. acetobutylicum* and *C. tetani* (Bruggemann et al., 2003). An interesting finding was the presence of 35 genes involved in sodium ion bioenergetics in *C. tetani*, in addition to a number of virulence genes such as toxin-, collagenase-, and adhesion protein genes. There have been a lot of advances in the genetics of clostridia in the past decade and, given continued interest in their capabilities as pathogens or alcohol producers, these advances should continue.

Industrial Applications

A description of the industrial application and medical importance of the species belonging to the Clostridiaceae (even for the species belonging to *Clostridium* sensu stricto) would go far beyond the scope of this chapter. The reader is referred to specialized reviews and book chapters (Smith and Williams 1984; Ljungdahl et al. 1989a; Ljungdahl et al. 1989b; Rood et al., 1997; Sussman 2001; Dürre, 2004; de la Maza et al., 2004).

Best known among industrial applications from the past is probably the acetone-butanol fermentation using *C. acetobutylicum* and various related strains. In the future, the application of clostridial and related strains will probably shift more to the use of recombinant enzymes, or products from them, expressed in different industrial expression vectors. An excellent historical review on the diversity of isolates and given names has been published by Jones and Keis (1995) and Johnson and Chen (1995) and provides an example of how pre-16S rRNA sequence analysis taxonomy and industrial interest led to confusion in the describing of species of clostridia.

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CHAPTER 1.2.21

Neurotoxigenic Clostridia

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Introduction

Certain bacterial species of the genus *Clostridium* are characterized by their ability to produce extremely potent neurotoxins: tetanus neurotoxin (TeNT) and botulinum neurotoxin (BoNT). TeNT inhibits neurotransmitter release of synapses of the central nervous system (CNS) causing the spastic paralysis of tetanus; BoNT inhibits the release of acetylcholine at peripheral cholinergic nerve terminals causing the flaccid paralysis of botulism. To date, one TeNT and seven (A–G) serologically distinct BoNTs are known (Schiavo et al., 2000).

Clostridium Tetani

Morphological and Cultural Characteristics

Tetanus neurotoxin (TeNT) is produced by a uniform group of bacteria belonging to the *Clostridium tetani* species. These bacteria are usually 0.3–0.6 μm in width and may vary considerably in length between 3 and 12 μm. They are Gram positive in young cultures, but they lose the Gram coloration upon prolonged incubations. *Clostridium tetani* is usually highly motile by peritrichous flagella, a property responsible for the swarming growth on agar medium, though some strains have no flagella. *Clostridium tetani* sporulates by forming translucid terminal enlargements, which give the typical drumstick appearance (*Clostridium* in Latin). The sporulation rate varies with the nature of the culture medium, strains, pH and temperature; sporulation does not take place above 41°C and at pH < 6 (Bytchenko, 1981). Spores are inactivated within one h at 100°C.

Germination of *C. tetani* spores occurs both under anaerobic and aerobic conditions, but the outgrowth of *C. tetani* is strictly dependent upon a low oxidation-reduction potential (Smith and Williams, 1984). *Clostridium tetani* is strictly anaerobic, and motile strains swarm over the entire surface of the agar leading to a transparent film. Discrete colonies (2–5 mm) can be obtained with media containing 3–4% agar. On blood agar, colonies are slightly raised, semitranslucent, gray, with an irregular margin and surrounded by a narrow zone of hemolysis. *Clostridium tetani* grows fairly well on the usual media containing peptones or tissue extracts.

Most of the usual biochemical tests used for *Clostridium* identification are negative, as no carbohydrates are acidified, and there is no proteolysis, and no lipase or lecithinase production. Gelatin is liquefied slowly (2–7 days) with production of H_2S and indole (Smith and Williams, 1984).

Genetic Characteristics

The *Clostridium* genus encompasses more than 100 species that display a wide range of phenotypes and genotypes (Hippe et al., 1992). *Clostridium tetani* has been classified in group II on the basis of 23S rRNA homology (Johnson and Francis, 1975).

The complete genome sequence of a toxigenic *C. tetani* strain consists of a 2,799,250-bp chromosome containing 2372 putative genes and a 74,082-bp plasmid, containing 61 genes (Brüggemann et al., 2003). *Clostridium tetani* possesses many genes for peptidases and for amino acid and lipid degradation, whereas genes for sugar utilization are lacking. It contains numerous transport-related genes; in particular, 35 genes for sodium ion-dependent systems indicate that Na^+ gradient is a major driving force in membrane transport. The TeNT encoding gene and seven putative regulatory genes are localized on the plasmid, whereas the tetanolysin (an hemolysin) gene and putative adhesin genes are located on the chromosome. Many genes encoding for putative adhesins have been identified: 2 fibronectin-binding proteins, 11 related surface-layer proteins, 19 homologues to a *Clostridium difficile* adhesin, and 2 proteins with multiple leucine-rich repeat domains similar to the *Listeria monocytogenes* internalin A (Brüggemann et al., 2003).

Clostridia Producing Botulinum Neurotoxins

BoNT-producing *Clostridia* display heterogeneous bacteriological characters and are divided

into several species and groups. The taxonomic position of the *C. botulinum* species was originally based on the production of a BoNT, and nontoxic variant strains, although genetically related to *C. botulinum*, were assigned to different species. Moreover, there are seven different serologically distinct BoNTs termed "A" to "G." *Clostridium botulinum* strains were divided into four physiological groups (Smith and Williams, 1984). Group IV includes *C. botulinum* G strains, which have been assigned to a different species termed "*C. argentinense*" (Suen et al., 1988).

BoNT can be produced by other *Clostridium* species such as *C. butyricum* and *C. baratii*, which have been implicated in human botulism. The toxinogenic property of the *Clostridium* strains remains an important characteristic, mainly in clinical aspects, but this single property is not sufficient to define a bacterial species. Moreover, toxigenicity is unstable, and it appears to be transferable to a wide variety of *Clostridium* species (Hatheway, 1990).

Morphological Aspects

BoNT-producing *Clostridium* are usually straight to slightly curved rods, 0.6–2 μm wide and 2–22 μm long. They are usually motile and have peritrichous flagella, except *C. baratii*, which is nonmotile. Spores are oval and swell one terminus of the cell. Type G strains sporulate poorly, and *C. butyricum* spores are central to subterminal and usually do not swell the cell (Cato et al., 1986).

These bacteria grow well in usual anaerobic liquid media with production of gas. Surface colonies can be grown on blood agar plates, incubated under anaerobic conditions; they can be circular (1–6 mm in diameter) or irregular, with a scalloped or lobate margin, translucent to semiopaque, and gray-white. A narrow zone of partial hemolysis surrounds colonies of BoNT-producing *Clostridium*, except colonies of *C. butyricum*, which is nonhemolytic.

Physiological Characters

Group I (*C. botulinum* A and proteolytic strains of types B and F) is characterized by proteolytic activity and by the nonacidification of carbohydrates. The optimal temperature of growth is 37°C. The thermoresistance of the spores is usually high (above 120°C; Smith and Williams, 1984).

Group II (*C. botulinum* E and glucidolytic strains of types B and F) acidifies many carbohydrates and produces mainly butyric and acetic acids from trypticase-yeast extract-glucose broth. They hydrolyze gelatin, but they are nonproteolytic (Cato et al., 1986). They have a lower

optimal temperature of growth (around 25–30°C), and they can grow and produce toxins at very low temperature. Spores are only moderately resistant to heat (not withstanding 10 min at 90°C; Smith, 1992).

Group III (*C. botulinum* C and D) is poorly proteolytic and is rather heterogeneous with respect to sugar fermentation and production of indole and hydrogen sulfide (Oguma et al., 1986). These bacteria grow at higher temperature than the other *C. botulinum* strains (30–37°C optimal range), with most strains growing well up to 45°C (Cato et al., 1986). Heat resistance of spores is intermediate.

Group IV (*C. argentinense*) is proteolytic, hydrolyzes gelatin, and does not ferment any of the usual carbohydrates. In contrast to the other strains of the *C. botulinum* groups, *C. argentinense* strains do not produce a lipase. The optimum temperature of growth is 30–37°C. Phenotypic differences have been observed between toxic and nontoxic strains by using cellular fatty analysis and multilocus enzyme electrophoresis (Ghanem et al., 1991).

Clostridium butyricum toxigenic strains are nonproteolytic, do not hydrolyze gelatin, and acidify strongly various carbohydrates, but they do not produce lipase or lecithinase (Smith, 1992). Toxigenic and nontoxigenic *C. baratii* strains display similar morphological and biochemical characteristics and are phenotypically related to *C. perfringens*. They produce a lecithinase but not a lipase. *Clostridium baratii* is readily differentiated from *C. perfringens* by its lack of hydrolysis of gelatin (Cato et al., 1986).

Genetic Characteristics

All the BoNT-producing *Clostridium* strains belong to the 23S rRNA homology group I (Johnson and Francis, 1975), which also includes *C. butyricum*. The genome sequence of *C. botulinum* A strain Hall is completed Sanger Microbes Project website (http://www.sanger.ac.uk/Projects/Microbes/). The chromosomal genome (3,888,916 bp) is larger than that of *C. tetani*. In addition to the botulinum locus, this strain contains two putative hemolysin genes on the chromosome and a small plasmid (16 kbp) containing a bacteriocin gene.

Genetics of Clostridial Neurotoxins

Neurotoxin Genes

The genes encoding the neurotoxins and associated nontoxic proteins (ANTP) have been cloned and sequenced in representative clostridial strains of each BoNT type (Popoff and

Eklund, 1995). The neurotoxin and ANTP genes are clustered (botulinum or tetanus neurotoxin locus). The organization of the botulinum locus is conserved in the 3′ part but differs slightly in the 5′ part in the different types of BoNT-producing *Clostridia*. The *bont* genes are preceded by the genes of the nontoxic nonhemagglutinin (NTNH) components. The *ntnh* and *bont* genes are transcribed in the same orientation (Fig. 1), whereas the hemagglutinin (HA) genes (*ha*33, *ha*17 and *ha*70) are transcribed in the opposite orientation. The *ha* genes are missing in the nonhemagglutinating toxinotypes A2, E and F. The *ha* genes of *C. botulinum* G only comprise *ha*17 and *ha*70. In toxinotypes A, E and F, a gene (*p47*) encoding a 47-kDa protein is immediately upstream of the *ntnh* gene, and both genes are transcribed in the same orientation. In addition, two genes (*orfX1* and *orfX2*), which are not related to *ha*, lie upstream of *p47*. A gene (*botR*, previously called "*orf*21" or "*orf*22") encoding a 21–22-kDa protein, which presents features of a regulatory protein, is present in different positions in different strains of *C. botulinum* (Fig. 1). In *C. tetani*, a gene (*tetR*) equivalent too *btR* is upstream of the *tetx* gene. No *antp*-like genes have been identified in *C. tetani* (Brüggemann et al., 2003).

Usually, one clostridial strain produces only one type of neurotoxin and the botulinum locus is present in a single copy on the genome; however, some rare strains synthesize two different BoNTs: BoNT/A-BoNT/B, BoNT/A-BoNT/F, and BoNT/B-BoNT/F (Lin and Johnson, 1995; Hutson et al., 1996; Henderson et al., 1997); the A-B strain contains two bont genes related to those of *C. botulinum* A and proteolytic *C. botulinum* B, respectively (Fujinaga et al., 1995). In such strains, the two neurotoxins are usually produced in different proportions (Henderson et al., 1997). Clostridial strains containing silent neurotoxin genes have been detected (Franciosa et al., 1994; Hutson et al., 1996; Jovita et al., 1998).

Genomic Localization and Transfer

The genes encoding for the different types of BoNT are present on different genetic elements, including phages, plasmid or chromosomes depending on the species and strain of *Clostridia* (Minton, 1995). These genes are linked to genetically mobile elements allowing for a loss or acquisition of neurotoxigenicity. In *C. tetani* and *C. argentinense*, the neurotoxin gene is present within a large plasmid, and in *C. botulinum* A, B, E and F, the toxin genes
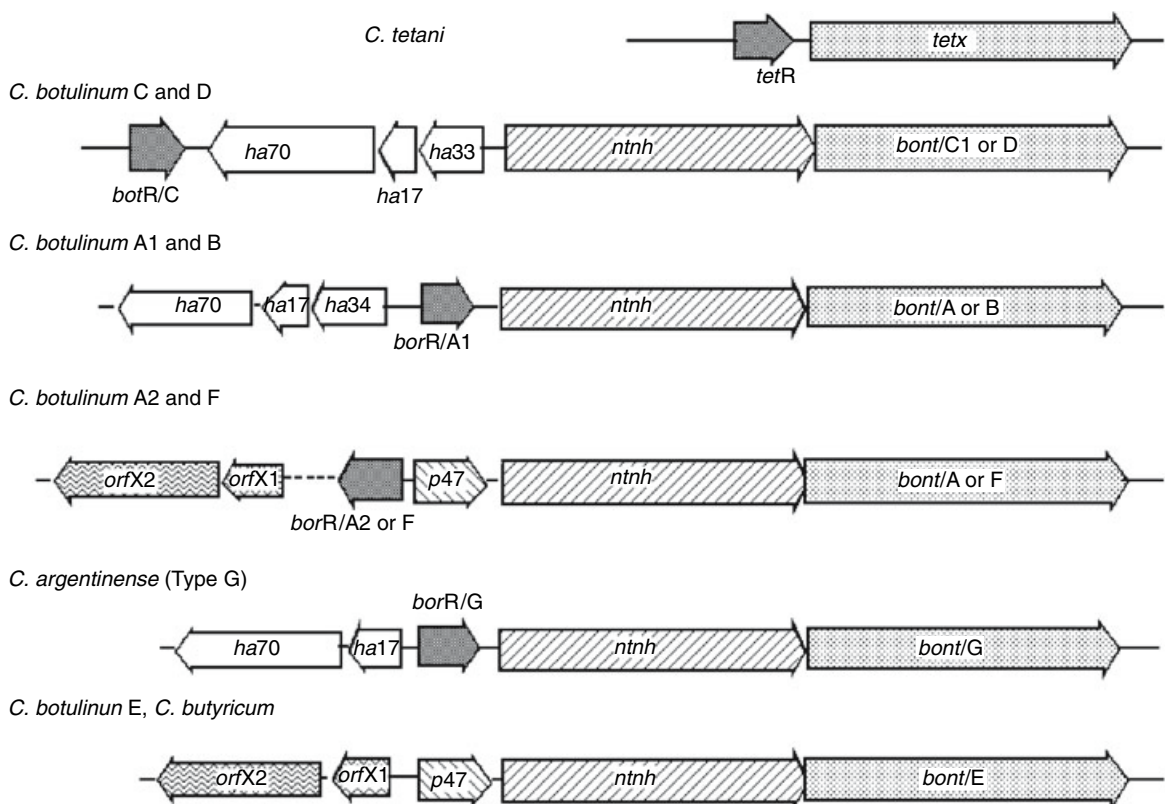
Fig. 1. Genetic organization of the botulinum and tetanus locus in *C. tetani* and in the different *C. botulinum* toxinotypes.

are located on the chromosome (Popoff and Eklund, 1995). *Bont/C* and *bont/D* are present within bacteriophages and their genes can be transferred in different *Clostridium* host strains determining their toxinotypes; indeed, transposable elements have been identified in *C. botulinum* C and D (Popoff and Eklund, 1995). A pseudolysogenic relationship corresponding to the presence of bacteriophages free within the bacterial cytoplasm exists between these phages and hosts. Thus, variants free of bacteriophages can be obtained with high frequency using curing reagents such as acridine orange and ultraviolet light. Under laboratory culture conditions, a proportion of the bacteria (depends on the strain and growth conditions [temperature and salinity]) is lysed and loses free bacteriophages that can reinfect them. Such lysogeny and reinfection cycles occur probably in the environment (soil and intestinal tract of birds and animals) and account for the isolation of nontoxigenic or low toxin producer variants (Eklund and Dowell, 1987).

In *C. botulinum* A and F, the involvement of bacteriophages in neurotoxin gene transfer has been suggested on the basis of the identification of a gene (*lyc*) in the vicinity of the *bont* genes. Since lytic enzymes participate in the bacteriophage life cycle, this could indicate that the botulinum locus is part of an integrated prophage. In *C. botulinum* A strain Hall, the *botulinum* locus is flanked by two insertion sequences in the 5′ part and by a transposase gene in the 3′ part. The *bont/E* gene has been transferred from a neurotoxigenic *C. butyricum* strain to a nontoxigenic *C. botulinum* E strain following a protocol resembling transduction with a defective phage (Zhou et al., 1993) suggesting that the reverse transfer may take place as well (Poulet et al., 1992).

The similarity among the different BoNTs and with TeNT, the fact that different *Clostridium* species can produce BoNT and that some strains contain combinations of *bont* genes (Franciosa et al., 1997; Henderson et al., 1997) strongly suggest that *bont* and *tetx* genes derive from a common ancestor and have been transferred between *Clostridium* strains.

Structure and Mode of Action of Clostridial Neurotoxins

The seven botulinum neurotoxins (BoNTs, types A–G) and the single tetanus neurotoxin (TeNT) are responsible for the clinical manifestations of botulism and tetanus, respectively. BoNTs bind to, enter peripheral cholinergic terminals, and cause a sustained block of acetylcholine release,

with ensuing flaccid paralysis and loss of function of autonomic glands. By contrast, TeNT reaches the CNS by retrograde axonal transport inside the axons of motor neurons and blocks neurotransmitter release at the inhibitory interneurons of the spinal cord, resulting in a spastic paralysis.

These neurotoxins are metalloproteases and exert their enzymatic action in the cell cytosol on selected proteins that form the core of the neuro-exocytosis machinery. As a result of such double specificity of binding (presynaptic membrane and substrate) and of their catalytic activity, these neurotoxins are the most potent toxins known, with a mouse 50% lethal dose (LD_{50}) between 0.1 ng and 1 ng of toxin per kg of body weight. Such extremely low values are expected to be even lower in the wild, where even a very small deficit in mobility may be sufficient to impair the survival of intoxicated animals. Different animal species show a large range of sensitivity to TeNT and to the different BoNTs. Humans and horses are even more sensitive to these neurotoxins than mice, whereas rats, birds, snakes and amphibians are rather resistant to TeNT, and turtles are insensitive (Payling-Wright, 1955; Gill, 1982).

Toxin Structure

The similar effect of the eight clostridial neurotoxins (CNTs) on nerve terminals is the result of a closely related protein structure. They are synthesized in the bacterial cytosol as a 150-kDa polypeptide chain without a leader sequence and are released in the culture medium only after bacterial autolysis. No protein is associated with TeNT, whereas the BoNTs are released in the form of multimeric complexes with a set of nontoxic proteins (Minton, 1995; Inoue et al., 1996). Some BoNT-associated proteins have hemagglutinating activity: HA of 17 kDa (HA17), HA of 34 kDa (HA34) and HA of 71 kDa (HA71). In addition, a large nontoxic, nonhemagglutinating protein of 139 kDa (NTNH) is always present, and it has been suggested that it nests the formation of the BoNT-NTNH complex, which may or may not progress to the formation of larger complexes. In fact, three forms of progenitor toxins have been characterized: extra-large size (LL sediments at 19S, approximately 900 kDa); large size (L sediments at 16S, 500 kDa), and medium size (M, sediments at 12S, 300 kDa). Complexed BoNTs are more stable than isolated BoNTs to proteolysis and denaturation induced by temperature, solvent removal, or acid pH, and to lyophilization (Sakaguchi, 1983; Chen et al., 1998). Toxins are adsorbed during their passage across the gastrointestinal tract and have been shown to transcytose across polarized epithelial mono-

layers (Maksymowych and Simpson, 1998; Simpson, 2000).

The single chain 150-kDa neurotoxins are inactive and are activated by specific proteolysis within a surface exposed loop subtended by a highly conserved disulfide bridge (Fig. 2). Several bacterial and tissue proteinases are able to generate the active di-chain neurotoxin. The heavy chain (H, 100 kDa) and the light chain (L, 50 kDa) remain associated via noncovalent interactions and via the conserved interchain S-S bond, whose integrity is essential for neurotoxicity (Schiavo et al., 1990; De Paiva et al., 1993).

The length of the polypeptide chains of CNTs varies from the 1251 amino acid residues of *C. butyricum* BoNT/E to the 1297 residues of BoNT/G and the 1315 residues of TeNT (Niemann, 1991). The exact length of the L and H chains depends on the site of proteolytic cleavage within the exposed loop. The H chains vary in size from the 829 amino acid residues of BoNT/E to the 857 residues of TeNT. The L chains range in size from the 419 amino acid residues of BoNT/E to the 449 residues of TeNT (Fig. 2), and its most conserved portions are the amino-terminal one hundred residues and the central region (residues 216–244; numbering of TeNT), containing the His-Glu-Xaa-Xaa-His binding motif of zinc-endopeptidases (where "Xaa" indicates any amino acid; Fig. 2).

The crystallographic structures of BoNT/A and BoNT/B and of the carboxy-terminal TeNT domain (Hc) reveal the presence of three distinct 50-kDa domains: a) an N-terminal domain endowed with zinc-endopeptidase activity (L chain), b) a membrane translocation domain (H_N) characterized by the presence of two 10 nm-long α-helices, which are reminiscent of elements present in colicins and in the influenza virus hemagglutinin, and c) a binding domain (H_C), composed of two subdomains, termed "H_C-N" and "H_C-C" (Umland et al., 1997; M. Knapp and B. Rupp, Protein Data Bank accession number 1A8D, 1998; Lacy et al., 1998; Swaminathan and Eswaramoorthy, 2000; Fig. 3).

This structural organization is functionally related to the four-step mechanism of neuron intoxication by CNTs, consisting of: 1) binding, 2) internalization, 3) membrane translocation, and 4) enzymatic target modification (Montecucco et al., 1994; Montecucco and Schiavo, 1995; Fig. 4). The L chain is responsible for the intracellular catalytic activity, the amino-terminal 50-kDa domain of the H chain (H_N) is implicated in membrane translocation, and the carboxy-terminal part (H_C) is mainly responsible for the neurospecific binding.

THE BINDING DOMAIN The H_C binding domain of these CNTs consists of two distinct subdo-mains, the N-terminal half (H_C-N) and the C-terminal half (H_C-C), with few protein-protein contacts between them. H_C-N is similar to some legume lectins, which are carbohydrate-binding proteins. The amino acid sequence of this subdomain is highly conserved among CNTs suggesting a very similar folding. The Hc-C subdomain contains a modified β-trefoil folding motif present in several proteins involved in recognition and binding functions such as IL-1, fibroblast growth factor, and the Kunitz-type trypsin inhibitors. Its sequence is poorly conserved among CNTs. It harbors one binding site for the oligosaccharide portion of polysialogangliosides in BoNT/A and /B, while the H_C-C of TeNT has two such sites (Rummel et al., 2003a; Rummel et al., 2003b).

THE TRANSLOCATION DOMAIN The H_N portions of BoNT/A and /B are very similar, and their sequences are highly homologous among the various CNTs. H_N has a cylindrical shape determined by the presence of a pair of unusually long and twisted 10-nm long α-helices, corresponding to segment 685–827 of BoNT/A (Lacy et al., 1998; Swaminathan and Eswaramoorthy, 2000; Fig. 3). At both ends of the pair, there is a shorter α-helix, which lies parallel to the main helices, and several strands packed along the two core helices. The overall structure of H_N resembles that of some viral proteins that undergo an acid-driven conformational change (Bullough et al., 1994; Weissenhorn et al., 1997).

THE CATALYTIC DOMAIN The metalloprotease domain has little similarity with related enzymes of known structure, apart from the α-helix segment including the zinc-binding motif (Fig. 3). The active site zinc atom is coordinated by the imidazole rings of the two histidines of the motif, a water molecule bound to the glutamic acid of the motif and a glutamic acid, which is conserved among clostridial neurotoxins. The Glu residue of the motif is particularly important because it coordinates the water molecule directly implicated in the hydrolytic reaction of proteolysis. Its mutation leads to complete inactivation of these neurotoxins (Li et al., 2000). The active site is similar to that of thermolysin and identifies a primary sphere of residues essential to the catalytic function, which coincides with the zinc coordinating residues. In addition, a secondary layer of residues, less close to the zinc center (including Arg362 and Tyr365 [numbering of BoNT/A]), appears to be directly involved in catalysis by providing transition state stabilization (Rigoni et al., 2001; Rossetto et al., 2001a; Binz et al., 2002). The active site of BoNTs is long and deep and shielded by the H chain; it becomes accessible to the substrate upon reduction of the interchain
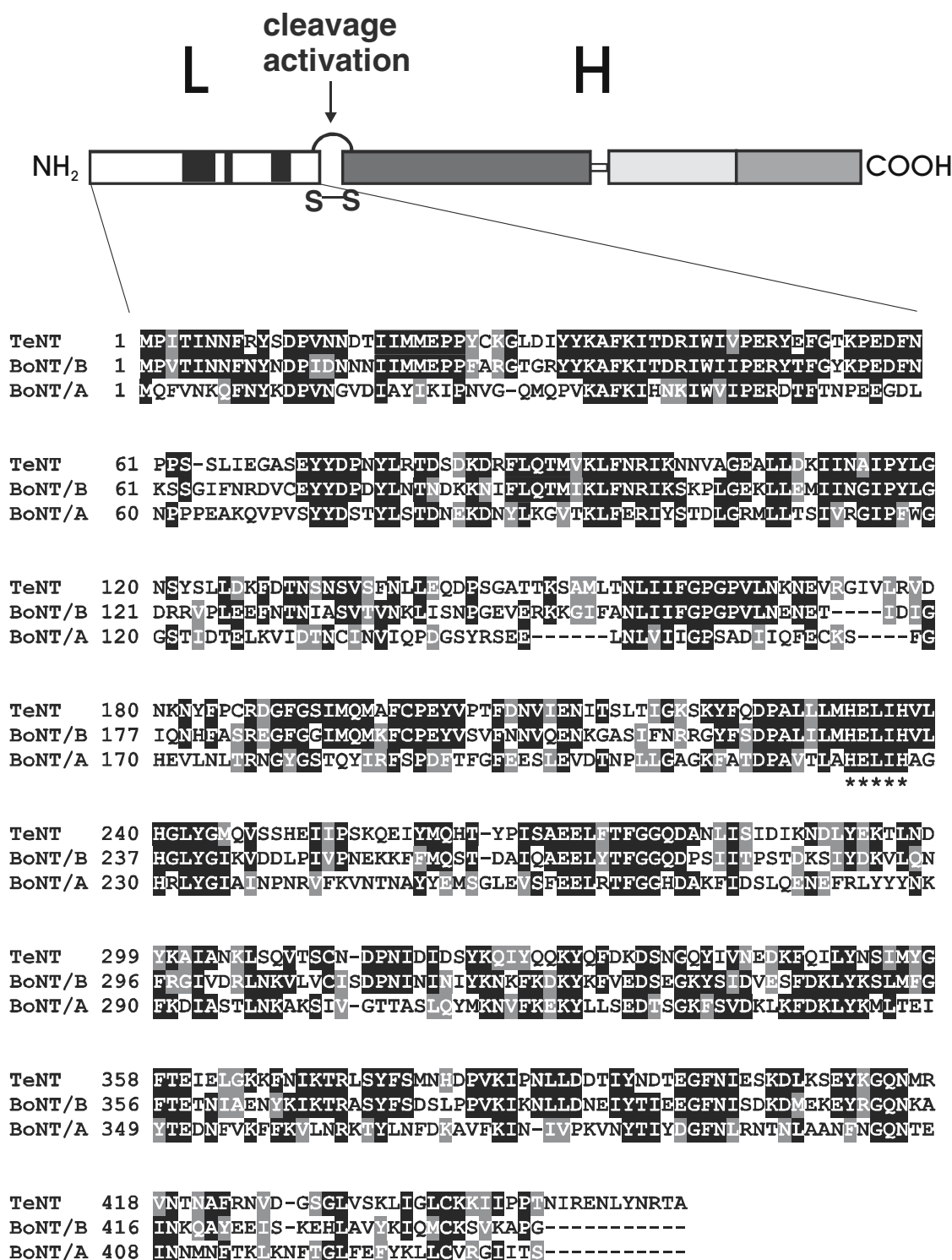
Fig. 2. Schematic structure of clostridial neurotoxins (CNTs) and amino acidic sequence of tetanus neurotoxin (TeNT), botulinum neurotoxin (BoNT)/B and BoNT/A L-chains. The neurotoxins are synthesized in the bacterial cytosol as inactive 150-kDa proteins and are activated by specific proteolysis within a surface exposed loop. These toxins are composed of a heavy chain (H, 100 kDa) and a light chain (L, 50 kDa), which remain associated via noncovalent interactions and via the conserved interchain S-S bond. The light chain is a zinc-endopeptidase acting in the neuron cytosol. The central region of the L chain (residues 216–244, numbering of TeNT) contains the His-Glu-Xaa-Xaa-His binding motif of zinc-endopeptidases (indicated by asterisks).

Fig. 3. Three-domain structure of clostridial neurotoxins. The crystallographic structure of botulinum neurotoxin (BoNT)/A highlights the three 50 kDa-domain structure, with the zinc atom (red ball) held in the center of the active site of the L chain (Lacy et al., 1998). The α-helix containing the HEXXH motif is shown in orange. The H_N domain is characterized by two 10 nm long pair of α-helices and is responsible for the membrane translocation of the L chain into the neuronal cytosol. The C-terminal H_c domain consists of two equally sized subdomains. The N-terminal one (H_{CN}) has two seven-stranded β-sheets similar to that of sugar binding proteins. The C-terminal one (H_{CC}) has a β-trefoil fold similar to that of the K^+ channel specific snake dendrotoxin. This part harbors the polysialoganglioside binding site of BoNT/A.
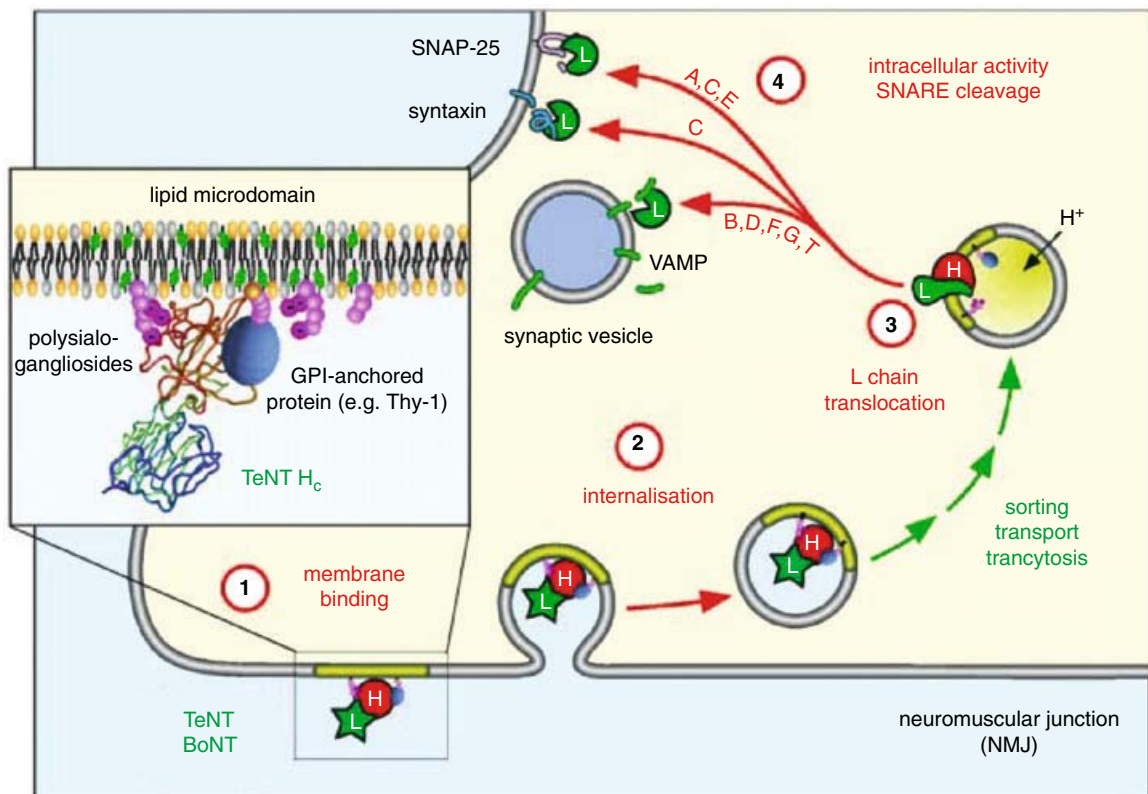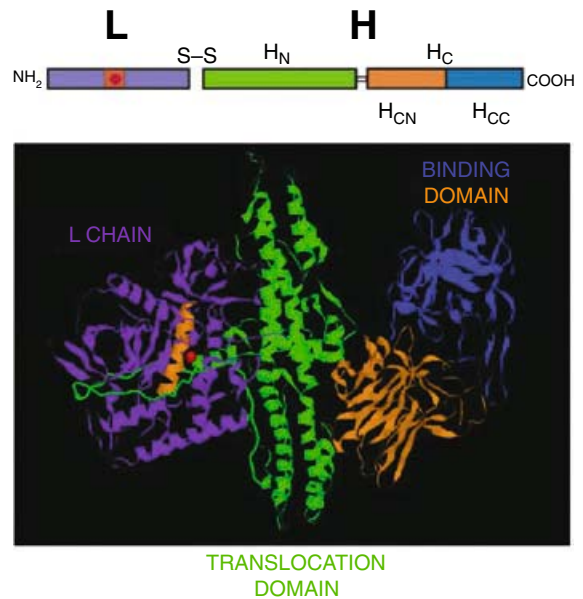
Fig. 4. Current view of tetanus neurotoxin (TeNT) and botulinum neurotoxins (BoNTs) entry at nerve terminals. BoNTs and TeNT bind to multiple receptors present within lipid microdomains of the presynaptic membrane of peripheral nerve terminals via the Hc domain. TeNT Hc is shown to interact with a glycosylphosphatidylinositol (GPI)-anchored protein (Thy-1) and to bind to two molecules of polysialogangliosides present within lipid microdomains in neurospecific binding that may be implicated in its internalization (see the inset). Binding is followed by internalization inside endocytic vesicles, whose lumen becomes acid following the activity of an ATPase proton pump. At low pH, BoNTs change conformation, insert into the lipid bilayer of the vesicle, and translocate the L chain into the cytosol. By contrast, TeNT is internalized and transported retroaxonally to the spinal cord (not shown) where it enters the inhibitory interneuron terminals as depicted here for the BoNTs at cholinergic peripheral terminals. Inside the cytosol, the L chain catalyzes the proteolysis of one of the three soluble *N*-ethylmaleimide sensitive factor-attachment protein receptor (SNARE) proteins. The capital letters indicate the serotype of the toxin acting on different targets.

disulfide bridge, and it appears to be capable of accommodating a 16-residue-long segment of the substrate.

Mode of Action of Clostridial Neurotoxins

NEUROSPECIFIC BINDING (STEP 1 OF FIG. 4) From the site of production or adsorption (intestine or wounds), BoNTs and TeNT diffuse in the body fluids. BoNTs bind with high affinity and specificity to the presynaptic membrane of skeletal and autonomic cholinergic nerve terminals; in addition, TeNT also binds to sensory and adrenergic neurons (Habermann and Dreyer, 1986). The H_C domain plays a major role in neurospecific binding (Lalli et al., 1999), but additional regions may be involved in determining the remarkable specificity for cholinergic terminals of CNTs. There is strong evidence that polysialogangliosides (particularly G_{D1b}, G_{T1b} and G_{Q1b}) are involved (Habermann and Dreyer, 1986; Halpern and Neale, 1995) together with proteins of the presynaptic membrane (Montecucco, 1986). The gangliosides binding site(s) have been localized within the C-terminal half of H_C (H_C-C), which harbors one binding site for the oligosaccharide portion of polysialogangliosides in BoNT/A and /B, while the H_C-C of TeNT has two such binding sites (Rummel et al., 2003a, Rummel et al., 2003b, and references cited therein). In addition, the Thy-1 glycoprotein participates in the binding of TeNT to spinal cord motoneurons (Lalli et al., 2003) and synaptotagmins I and II (membrane-bound Ca^{+2}-signaling machines) in that of BoNT/B, together with gangliosides (Nishiki et al., 1996; Yowler et al., 2002; Dong et al., 2003; Rummel et al., 2004).

Both TeNT and BoNTs bind to the presynaptic membrane of α-motoneurons, but they then follow different intracellular trafficking paths. BoNTs block neuroexocytosis at peripheral terminals, whereas TeNT causes the same effect on CNS synapses of the spinal cord. These different final destinations of TeNT and BoNTs must be determined by specific coreceptors that drive them into different intracellular routes. The presence of both glycolipid and glycoprotein coreceptors is in keeping with a high affinity and specific binding of CNTs to the presynaptic membrane because multiple interactions are involved. However, the receptor molecules identified so far are not cholinergic specific, and the CNT regions and coreceptors responsible for this specificity and for steering BoNTs to the inside of cholinergic terminals and TeNT to the spinal cord via retroaxonal transport remain to be identified.

INTERNALIZATION (STEP 2 OF FIG. 4) Since the L chains of CNTs block neuroexocytosis by acting in the cytosol, at least this toxin domain must reach the cell cytosol. All available evidence indicate that CNTs do not enter the cell directly from the plasma membrane, rather they are endocytosed inside acidic intracellular compartments via a temperature- and energy-dependent process (Dolly et al., 1984; Matteoli et al., 1996). A protein coreceptor of TeNT is thought to be responsible for its inclusion in an endocytic vesicle, which moves in a retrograde direction along and inside the axon (Schwab and Thoenen, 1976; Herreros and Schiavo, 2002), whereas BoNTs protein coreceptors guide them inside vesicles that acidify within the cholinergic terminal. The TeNT-carrying vesicles reach the cell body of the motoneuron in the spinal cord and then move to dendritic terminals to release the toxin in the intersynaptic space. TeNT equilibrates between pre- and postsynaptic membranes and then binds and enters the inhibitory interneurons of the spinal cord most likely via synaptic vesicle endocytosis (Matteoli et al., 1996).

MEMBRANE TRANSLOCATION (STEP 3 OF FIG. 4) To reach the cytosol, the L chain must cross the hydrophobic barrier of the vesicle membrane, and the acidity of the lumen is essential for such a movement. CNTs have to go through a low pH step for nerve intoxication to occur (Simpson et al., 1994; Williamson and Neale, 1994). This causes a conformational change from a water-soluble "neutral" structure to an "acid" structure, with the surface exposure of hydrophobic patches, which mediate the interaction of the H and L chains with the hydrocarbon core of the lipid bilayer (Montecucco et al., 1989). Following this low pH-induced membrane insertion, BoNTs and TeNT form transmembrane ion channels in planar lipid bilayers (Koriazova and Montal, 2003, and references cited therein) and in cell membranes (Beise et al., 1994; Sheridan, 1998). Membrane translocation of bacterial protein toxins (including CNTs) is the least understood step of their mechanism of action. For BoNT/A, a chaperone activity for the H chain has been envisioned preventing the aggregation of the L chain in the acid vesicle interior and preserving an unfolded conformation during translocation, release and refolding of the L chain at the neutral pH of the cytosol (Koriazova and Montal, 2003). The release of the L chain into the cytosol requires reduction of the interchain disulfide bond (Montecucco and Schiavo, 1995).

PROTEOLYSIS OF SNARE POTEINS (STEP 4 OF FIG. 4) Once in the cytosol, CNT L chains display their catalytic activity. CNTs are remarkably specific proteases that recognize and cleave only three proteins, which form the core of the neuroexocytosis machinery (Schiavo et al., 2000; Rossetto

et al., 2001b). Vesicle-associated membrane protein (VAMP), synaptosomal associated protein of 25 kDa (SNAP-25), and syntaxin form a heterotrimeric coiled-coil complex, which juxtaposes the vesicle to the target membrane (Sutton et al., 1998) and is involved in their fusion (Chen and Scheller, 2001). VAMP is a family of proteins located on the membrane of intracellular vesicles with a C-terminal tail facing the vesicle lumen, a single transmembrane domain, and the remaining N-terminal part exposed to the cytosol (Fig. 5). Different VAMP isoforms are located on different cell vesicles, and each VAMP helps direct each vesicle to its appropriate target membrane. VAMP-1 and -2 are the isoforms mainly involved in the binding and fusion of neurotransmitter containing synaptic vesicles with the presynaptic membrane (neuroexocytosis). Syntaxin is anchored to target membranes via a C-terminal hydrophobic tail. Of the many syntaxin isoforms presently known, syntaxin 1A, 1B and 2 are the main syntaxins involved in neuroexocytosis. SNAP-25 proteins (few isoforms) are bound to the target membrane via fatty acids covalently linked to cysteine residues present in the middle of the polypeptide chain.

TeNT, BoNT/B, /D, /F and /G cleave the vesicle-associated membrane protein VAMP/synaptobrevin, at different single peptide bonds; BoNT/C cleaves both syntaxin and SNAP-25 (two proteins of the presynaptic membrane), while BoNT/A and /E cleave SNAP-25 at different sites within the COOH-terminus (reviewed in Schiavo et al. [2000] and Humeau et al. [2000]; Fig. 6). Proteolysis of one soluble N-ethylmaleimide sensitive factor-attachment protein receptor (SNARE) protein prevents fusion complex formation, or it may result in a nonfunctional complex (Hayashi et al., 1994; Pellegrini et al., 1995; Humeau et al., 2000; Fig. 5).

Strikingly, TeNT and BoNT/B cleave VAMP at the same peptide bond (Gln76-Phe77; Table 1) and yet, when injected into the animal, they cause the opposite symptoms of tetanus and botulism, respectively (Schiavo et al., 1992a; Schiavo et al., 1992b). This conclusively demonstrates that the different symptoms of the two diseases derive from different sites of intoxication rather than from a different molecular mechanism of action.

The biochemical basis of the CNTs specificity for the three SNAREs is only partially known. Experimental evidence clearly indicates that CNTs recognize a nine-residue-long motif characterized by the presence of three carboxylate residues alternated with hydrophobic and hydrophilic residues (Rossetto et al., 1994; Pellizzari et al., 1996; Washbourne et al., 1997; Vaidyanathan et al., 1999). This motif is present in two copies (V1 and V2) in VAMP and syntaxin and in four copies in SNAP-25. In addition, TeNT recognizes a positively charged cluster of residues of VAMP located C-terminal to the cleavage site (Cornille et al., 1997).

The peptide bonds hydrolyzed by each neurotoxin are reported in Table 1. Apart from TeNT and BoNT/B, each of the different CNTs cata-
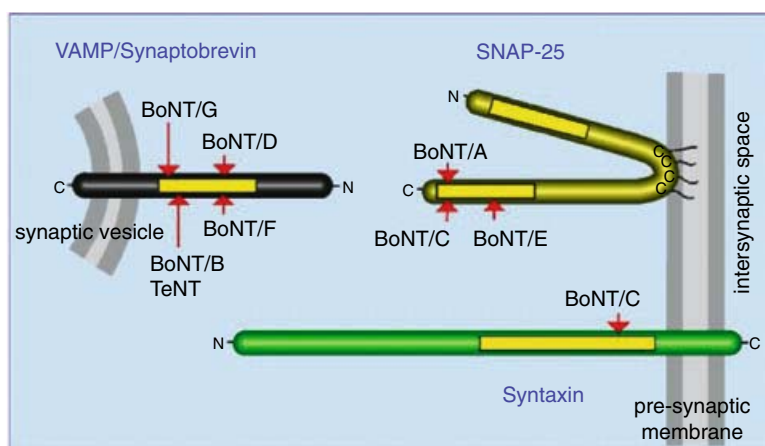
Fig. 5. Schematic of a nerve terminal showing the process of the neurotransmitter (NT) release (left) and the mechanism of action of tetanus and botulinum neurotoxins (right). Neurotransmitter containing vesicles are characterized by a transmembrane protein, vesicle-associated membrane protein (VAMP), that binds specifically two proteins of the cell membrane (syntaxin and synaptosomal associated protein of 25 kDa [SNAP25]). These three proteins make a complex that forces the vesicle and cellular membranes into close contact and eventually to fuse, thus releasing the NT into the intersynaptic space. Tetanus and botulinum neurotoxins are Zn-endopeptidases that cleave VAMP, or syntaxin or SNAP25, preventing the complex formation and therefore exocytosis of NT.
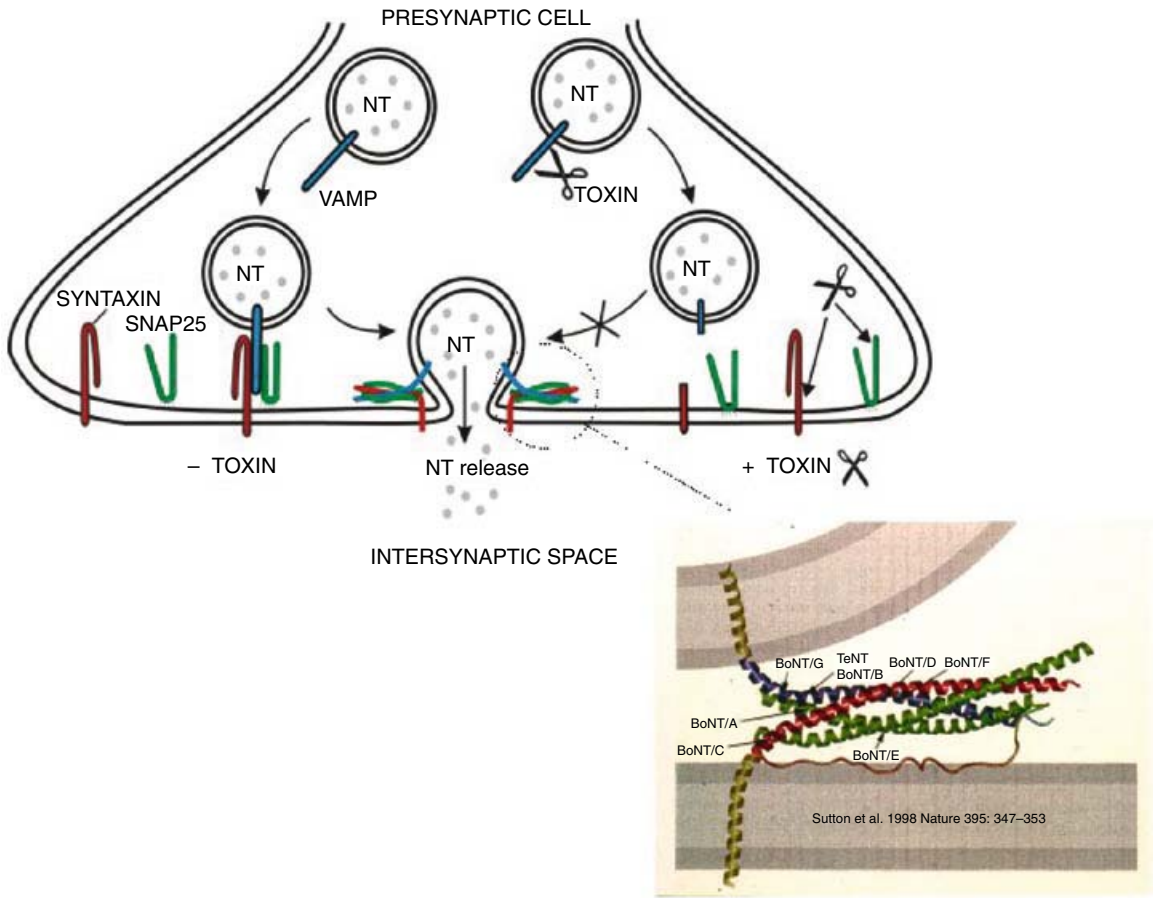
Fig. 6. Schematic structure of soluble *N*-ethylmaleimide sensitive factor-attachment protein receptor (SNARE) proteins and cleavage site of tetanus neurotoxin (TeNT) and botulinum neurotoxins (BoNTs). Vesicle-associated membrane protein (VAMP) has a short C-terminal tail protruding into the vesicle lumen and a trasmembrane segment, followed by a longer cytosolic part, which is highly conserved between isoforms and species, whereas the N-terminal portion is poorly conserved and rich in prolines. Syntaxin is inserted in the plasma membrane, and most of its mass projects into the cytosol. Synaptosomal associated protein of 25 kDa (SNAP-25) is bound to the cytosolic face of presynaptic membrane via palmitoylated cysteine residues and via interactions with syntaxin. Arrows indicate the sites of cleavage of clostridial neurotoxins (CNTs). The bottom-right panel shows the crystallographic structure of the soluble *N*-ethylmaleimide sensitive factor-attachment protein receptor (SNARE) complex.

Table 1. Tetanus and botulism neurotoxins target and peptide bond specificities.[a]

Toxin type	Intracellular target	Peptide bond cleaved
		P4-P3-P2-P1—P1′-P2′-P3′-P4′
TeNT	VAMP	Gly-Ala-Ser-Gln—Phe-Glu-Thr-Ser
BoNT/A	SNAP-25	Glu-Ala-Asn-Gln—Arg-Ala-Thr-Lys
BoNT/B	VAMP	Gly-Ala-Ser-Gln—Phe-Glu-Thr-Ser
BoNT/C	Syntaxin	Asp-Thr-Lys-Lys—Ala-Val-Lys-Phe
BoNT/C	SNAP-25	Ala-Asn-Gln-Arg—Ala-Thr-Lys-Met
BoNT/D	VAMP	Arg-Asp-Gln-Lys—Leu-Ser-Glu-Leu
BoNT/E	SNAP-25	Gln-Ile-Asp-Arg—Ile-Met-Glu-Lys
BoNT/F	VAMP	Glu-Arg-Asp-Gln—Lys-Leu-Ser-Glu
BoNT/G	VAMP	Glu-Thr-Ser-Ala—Ala-Lys-Leu-Lys

Abbreviations: TeNT, tetanus neurotoxin; BoNT, botulinum neurotoxin; VAMP, vesicle-associated membrane protein; SNAP-25, synaptosomal associated protein of 25 kDa; and SNARE, soluble N-ethylmaleimide sensitive factor-attachment protein receptor.

[a]Sequences refer to human SNAREs.

lyzes the hydrolysis of a different peptide bond. Thus CNTs are well-defined tools to probe the role of their targets in different cellular processes, and finer dissections of SNARE activities can be performed on the basis of the peptide bond hydrolyzed by the different CNTs. Moreover, since the three SNAREs are not cleavable by the CNTs when they are complexed (Hayashi et al., 1994; Pellegrini et al., 1995), these neurotoxins can be used to assay the state of SNARE assembly. Although BoNT/A removes only nine residues from the SNAP-25 C-terminus, neuroexocytosis is impaired, thus indicating that this part of the molecule plays a relevant role in exocytosis.

The cleavage of the SNARE proteins by the BoNTs within the nerve cells does not cause cell death, as do other bacterial toxins (diphtheria toxin, shiga toxins, etc.). Within a time period that varies with the type of nerve, animal species, and type of BoNT, the toxin is inactivated within the nerve and new SNARE protein is synthesized leading to full functional recovery. In other words, if enough toxin has been released within the body, the loss of function of respiratory muscles and other muscles leads to death. However, in the case of milder intoxications, or if the patient is kept alive by mechanical ventilation, with time the patient will fully recover.

Botulinum Neurotoxins in Human Therapy

The demonstration that BoNTs specifically inhibit peripheral cholinergic nerve terminals and that this is followed by a complete functional recovery of the neuromuscular junction have provided the scientific basis for the rapidly growing use of BoNTs in the therapy of a variety of human diseases caused by hyperfunction of cholinergic terminals (Scott, 1989; Jankovic and Hallet, 1994). Injections of minute amounts of BoNT into the muscle(s) to be paralyzed lead to a depression of the symptoms lasting months, depending on the type of toxin used. After the first demonstration of the efficacy of BoNT/A injection in the treatment of strabismus (Scott, 1989), the therapeutic use of this neurotoxin has been extended to dystonias and other movement dysfunctions that respond to treatment based on a partial paralysis of the neuromuscular junction (Table 2). More recently, the use of BoNTs has been extended to diseases of the autonomic cholinergic nerve system such as hyperhydrosis and hypersalivation and to myofascial pain and tension and migraine headaches (Moore and Naumann, 2003). There are comparatively few side effects of the treatment with BoNT/A; the

most important one is the production of antibodies that renders the patient resistant to the toxin. In general, this occurs with the large doses of toxin necessary to weaken strong muscles within anatomical areas rich in lymph nodes such as the neck, treated for spasmodic torticollis. If there are neutralizing anti-BoNT/A antibodies, one has to switch to a different serotype. BoNT type B has been recently introduced for the treatment of cervical dystonias (Brashear et al., 1999), and very encouraging results have been obtained with BoNT/C (Eleopra et al., 1997; Eleopra et al., 2002).

Ecology of Neurotoxin-producing *Clostridia*

Clostridium tetani, BoNT-producing *Clostridia*, and other *Clostridia* are largely present in the environment in the form of spores, which are able to survive for very long periods under extreme conditions (involving heat, dryness, radiation, chemicals and oxygen). Spore germination and cell division occur only under anaerobic conditions and in the presence of the appropriate nutritional requirements. This restricts the habitat of the *Clostridium* to anaerobic, or low oxygen, areas containing sufficient amounts of organic materials. On the basis of their physiological properties (tolerance or extreme sensitivity to oxygen, and requirement of particular pH, temperature and substrate for growth or spore germination), the distribution of the different *Clostridium* species in nature is not uniform (Hippe et al., 1992). Saccharolytic *Clostridia* such as *C. butyricum* are able to grow on carbohydrates and are mainly found in decomposing vegetables and fruits. Proteolytic and gelatinolytic *C. botulinum* and *C. tetani* are preferentially associated with animal cadavers and soils or sediments rich in organic material. They can also be found in the digestive tract of healthy humans and animals, and, after death, they participate in the cadaver's decomposition.

Clostridium tetani

This ubiquitous organism is commonly found in all parts of the world. The frequency of its isolation from soil samples varies (30–42%; Smith and Williams, 1984). This variability is due to pH, temperature, moisture, and amount and type of organic materials. Thus, germination and multiplication of *C. tetani* have been observed preferentially in neutral or alkaline soil, with temperatures >20°C and humidity reaching 15% (Smith and Williams, 1984). *Clostridium tetani* shows a higher presence in southern regions, and

Table 2. Therapeutic use of botulinum neurotoxins in humans.

Disease/condition	Clinical effect	Benefit	Duration (months)
Blepharospasm	E	3+	2–4
Hemifacial spasm	E	3+	2–6
Laringeal dysphonia	E	3+	1–6
Focal hyperhydrosis	E	3+	>12
Hypersalivation	E	3+	Several months
Oromandibular dystonia	E	2+	1–3
Torticollis	E	2+	1–3
Strabismus	E	2+	Up to many months
Limbs dystonia	E	2+	1–3
Occupational cramps	E	2+	1–3
Myokymia	E	2+	2
Facial synkinesia	X	2+	1
Pathological lacrimation	X	3+	<6
Esophageal achalasia	X	2+	1–2
Bruxism	X	+	1–5
Spasticity		+	1–3
Urinary retention	X	+	1–2
Essential tremor	X	+	1–3
Nystagmus	X	+/–	?
Dysphagia	X	+/–	Up to many months
Facial wrinkles	X	+/–	?
Myofacial pain-dysfunction	X	+/–	?
Muscle-contraction headache	X	+/–	?
Single injections			
Anal fissures	ND	ND	Very effective
Vaginism	X	ND	Very effective

Symbols and abbreviations: E, established; X, experimental; 3+, very beneficial; 2+, moderately beneficial; +, somewhat beneficial; +/–, barely beneficial; and ND, not determined.

accordingly the incidence of tetanus is higher in warmer countries than in the cooler parts of the world (Smith and Williams, 1984). This bacterium can be found in the intestine of animals but does not represent a significant part of the normal digestive flora. Different surfaces and objects contaminated with soil particles, dust or feces may contain *C. tetani*. Toxigenic strains have even been isolated within hospitals from catgut, cotton wool, dust and air samples, human skin, and wounds (Bytchenko, 1981).

BoNT-producing *Clostridia*

These organisms occur in most parts of the world in habitats that include soils as well as lake and sea sediments. However, the different toxinotypes of *C. botulinum* are not equally distributed, some being restricted to particular ecological areas. Table 3 summarizes the frequencies of isolation of the various toxinotypes in different countries. The factors responsible for the geographical distribution of the different toxinotypes are still poorly understood. Toxinotypes A and B occur more frequently in soil samples, whereas toxinotype E is more predominant in sea or lake sediments. Toxinotypes C and D appear to be obligate parasites of birds and other animals, and cadavers of people who died of botulism are a major source of these organisms. They are seldom encountered in soil samples, except in the areas where the incidence of animal botulism is high (Smith and Williams, 1984). *Clostridium botulinum* is not usually found in the digestive tract of healthy humans, but it can be found in that of animals, particularly *C. botulinum* C and D. *Clostridium butyricum* and *C. baratii* are very widespread in the environment and are present as well within animals (Cato et al., 1986); the first two neurotoxigenic *C. butyricum* strains were isolated from infant botulism (Aureli et al., 1986).

Botulinum Neurotoxins in Food

In general, the contamination of fish and other aquatic animals by *C. botulinum* reflects that of the sediments of the respective geographic areas. The incidence of *C. botulinum* depends on these areas and on the type of fish: gills and intestines are the most heavily contaminated parts. *Clostridium botulinum* E does not multiply in the intestine of living fish, but after death, the bacterium can grow and produce BoNT. Dead fish in the sediments contribute to the persistence and multiplication of *C. botulinum* in the aquatic environment (Dodds, 1993a) and to large

Table 3. Frequencies of isolation of the various toxinotypes in different countries.

Country	% Positive samples	Types %					
		A	B	C/D	E	F	G
Eastern United States soil	19	12	64	12	12	0	0
Western United States soil	29	62	16	14	8	0	0
United States Great Lakes and east coast (north of 36°) sediment, shore soil	26	0	1	6	93	0	0
United States west coast (north of 40°) sediment, shore soil	43	6	1	0	92	1	0
United States east coast (south of 40°) sediment	5	7	7	49	37	0	0
United States west coast (south of 36°) sediment	10	44	50	0	0	6	0
South America (Brazil, Paraguay, Argentina, and Falkland Islands)	28	33	33	13	0	20	1
Central Africa (Kenya)	25	89	0	11	0	0	0
South Africa	12	0	50	50	0	0	0
Indonesia and Java	12	5	17	73	5	0	0
Australia and New Zealand	29	50	0	50	0	0	0
Sweden, Norway, Denmark, Greenland, Iceland, and Baltic coast (shore soil and sediment)	43	3	2	0	95	0	0
Britain and Ireland (sediment, soil) Denmark, Iceland, and Netherlands (soil)	35	0	67	19	14	0	0
France and Italy (soil)	4	29	71	0	0	0	0
Switzerland	44	19	56	3	0	19	3
Georgia, Lithuania, Kazakhstan, Uzbekistan, Ukraine, Moldova, and Kyrgyz Republic, and Caspian Sea (sediment and soil)	15	8	18	2	72	0	0
China, Thailand, and Bangladesh (sediment and soil)	69	16	14	61	8	1	0
Japan (soil and sediment)							0
North part	12	0	0	0	99	1	
South part	28	0	0	92	8	0	

outbreaks of botulism caused by the ingestion of dead fish containing high titer of BoNT, producing an ever-increasing chain reaction of disease spread (Eklund et al., 1982).

Contamination of meat with *C. botulinum* is less frequent than that of fish, and toxinotypes A and B are usually predominant, followed by C, and more rarely by E. Fruits and vegetables may be contaminated by *C. botulinum* as a result of soil contamination. Honey may contain *C. botulinum*, but the level of contamination is usually low (1–10 spores per kg). However, honey samples associated with infant botulism contained as many as 10^3–10^4 spores per kg. The presence of *C. botulinum* in other foods (such as dairy products or prepared foods [boil-in-bag foods, vacuum-packed foods, pressurized foods, dehydrated and freeze-dried foods]) is very rare (Dodds, 1993a).

Tetanus

The Disease

The first record of tetanus coincides with the beginning of medical literature, when Hippocrates in the Greek isle of Kos described the case of a sailor who developed a spasmodic generalized contracture of skeletal muscles, which he termed "tetanus" (τεταηοσ in Greek means contraction). Generalized tetanus is the most common form of the disease and the portal of entry in 80% of cases is a minor wound or skin scratch. Between the time of injury and the first symptoms, there is a lag phase varying between few days up to four weeks. Tetanus usually begins with a characteristic facial trismus (lockjaw or "risus sardonicus") with difficulty in swallowing and neck stiffness (Bleck, 1989). With time, the muscle paralysis extends downwards to the muscles of the trunk, abdomen and legs. The typical tetanic seizure is characterized by a sudden burst of tonic contraction of skeletal muscles causing opisthotonos, flexion and adduction of the arms, and extension of the lower extremities. The seizure is very painful and can be triggered by minor stimuli such as a small light. Later on, autonomic symptoms develop including alterations of blood pressure and of the cardiac rhythm, and sweating. Glottal and laryngeal spasm may develop and cause cyanosis and asphyxia.

A milder form of the disease is local tetanus, with rigidity of the group of muscles close to the site of injury and spreading of TeNT. This local tetanus may persist for a considerable period without further developments or it may progress to generalized tetanus. It is due to dysfunction of interneurons that inhibit the alpha motor neu-

rons of the affected muscles without further spread through the CNS.

Owing to the benefit of vaccination with the very immunogenic tetanus toxoid (formaldehyde-treated and inactivated tetanus toxin), tetanus has almost disappeared from the more developed countries, but it still takes hundreds of thousand of lives in those regions of the world where vaccination is not practiced (Galazka and Gasse, 1995). Here, the major form of tetanus is tetanus neonatorum, which develops after the umbilical cord of babies born to nonimmunized mothers is cut. In fact, tools contaminated with spores are often employed and rags, often soiled with animal feces, are used as dressing, thus increasing the possibility of contamination.

Tetanus is often fatal; death follows body exhaustion and is usually due to respiratory or heart failure (Bleck, 1989). Mortality has decreased in the more developed parts of the world owing to modern intensive care techniques, but it is still high because of the usually advanced age of patients and because their respiration has to be mechanically assisted for long periods, which in turn risks development of pulmonary infections.

Epidemiology of Tetanus

Tetanus is almost invariably associated with infection of necrotic wounds. Tetanus from ingestion of preformed toxin in food or by absorption of tetanus toxin produced by *C. tetani* in the intestine has not been reported, though this organism has been found in human and animal feces (Smith and Williams, 1984), and it can grow and produce toxin in the intestinal tract of germ-free rats (Wells and Balish, 1983). As an anaerobic bacterium, *C. tetani* does not grow in the normal oxygenated tissue and some degree of tissue necrosis is necessary for spore germination and bacterial outgrowth. Concomitant infection by other bacteria may also provide conditions suitable for the growth of *C. tetani* (Smith and Williams, 1984).

Tetanus is frequent in subtropical and tropical countries with poor hygienic conditions and where antitetanus vaccination is not performed or the vaccination protocol is only partially achieved. Neonatal tetanus is currently the most represented form of tetanus in the world (Galazka and Gasse, 1995). The incidence of tetanus in countries where vaccination is a common practice is very low (annual incidence of 0.2 per million) and always involves aged persons whose level of anti-TeNT antibodies has fallen with age. A single booster injection in adulthood is sufficient to bring the level of antitoxin antibodies to

a fully protective level, and this procedure is highly recommended.

Domestic animals can contract tetanus, and the disease is frequent and severe in horses. The susceptibility to TeNT depends on the animal species. Birds are relatively resistant and cold-blooded animals quite resistant. The most susceptible animal species, in decreasing order, are horse, sheep, goat, mouse, rat, rabbit, monkey, guinea pig, dog, cat, pigeon, and chicken (Payling-Wright 1955; Dezfulian, 1989). The different susceptibility to tetanus can be attributed to many factors including variable amounts and nature of receptors in the peripheral and/or central nervous system. Environmental factors may also play a role. For example, frogs are much more resistant to TeNT at 15°C than at 23°C, most likely because endocytosis of the toxin is reduced by low temperature.

Botulism

Human Botulism

Unlike tetanus, botulism follows the ingestion of foods containing preformed BoNT, and very rarely is it caused by wound infections. Four forms of botulism are recognized according to the mode of acquisition.

FOODBORNE BOTULISM The cause is ingestion of food maintained under anaerobic conditions, which permit the growth of *C. botulinum* with the production of sufficient amounts of BoNT. This is the most common form of disease in adults. Usually, several persons are intoxicated at the same time by sharing contaminated food. Usually BoNT/A, /B and /E and much less frequently types F, C or D are involved (Hatheway, 1993b). BoNT are released as complexes with accessory nontoxic proteins, which greatly increase the resistance of BoNTs within the gut, where the toxin is adsorbed and enters the general circulation to reach its neuronal target sites (Maksymowych et al., 1999). All the symptoms of botulism can be ascribed to the blockade of the peripheral cholinergic nerve terminals (in both skeletal muscle and autonomic ganglia) with ensuing flaccid paralysis of the muscles and autonomic dysfunctions (Tacket and Rogawski, 1989). BoNTs affect initially head nerve terminals causing diplopia or visual blurring, ptosis, dysphagia, facial paralysis, reduced salivation and lacrimation. The paralysis then progresses down to the muscles of the trunk, including the respiratory, visceral and then limb muscles.

The seriousness of the illness depends on the amount and type of BoNT. Death follows the blockade of respiratory muscles, but if the

patient is mechanically ventilated, with time he will recover completely following the metabolism of the toxin and the resynthesis of intracellular substrates. In general, botulism is much less dangerous than tetanus because, in most cases, the amount of toxin that reaches the general circulation is not sufficient to block respiration; it is believed that most cases of BoNT intoxication fail to attain clinical relevance and go unnoticed. Type A botulism is more dangerous than types B and E, and its symptoms persist much longer.

The incidence and the types of botulism depend on the occurence of *C. botulinum* in the environment and subsequently in foods and in cooking practices. The geographical distribution of the different types of botulism corresponds to the distribution of the different toxinotypes of *C. botulinum* in the environment. Thus, type E botulism is mainly found in the colder regions of the northern hemisphere and aquatic animals are usually affected (Hauschild, 1993), and types A and B botulism occur generally in the temperate countries. Most botulism outbreaks are caused by home prepared or stored/fermented food, while contamination of foods prepared by food industries is very rare (Hauschild, 1993).

INFANT BOTULISM The cause is ingestion of spores of toxigenic *Clostridia* that germinate, multiply, and produce BoNT in the intestinal tract. This is possible only when the normal intestinal flora have not yet been established, and in fact most cases of infant botulism develop in babies (1–6 months of age at the onset; Arnon, 1989). The minimum infective dose for human infants has been estimated to be 10–100 spores (Arnon, 1989). Moderate to high BoNT levels ($10–10^5$ times the mouse lethal dose per g) and viable *C. botulinum* are recovered from stools of affected infants for a long period after the onset of the symptoms. Therefore, infant botulism is a toxo-infection rather than an intoxication (as in adult botulism). The affected infants present with constipation, weak sucking, hypotonia and ptosis. In the more severe cases, the patient becomes lethargic and loses head control. The disease progresses to a flaccid paralysis, which may extend to respiratory muscles with arrest. Again, type A is generally more dangerous than type B or E, and the recovery time is accordingly longer (Arnon, 1989). Infant botulism has been identified in many developed countries, but most cases were recorded in the United States, particularly in California. It is presumed that contamination occurs with *C. botulinum* spores from the environment, and honey feeding has been frequently implicated (Arnon, 1989).

A new category of botulism of undetermined etiology has been proposed to occur in patients older than 1 year of age and to which no association with food or wounds has been recognized as the source of toxin. *Clostridium botulinum* and its toxin have been evidenced in these patients' stools for a long period (3 weeks and in some cases up to 6 months; Hatheway, 1993a). It has been concluded that this form of botulism in adults (as in infant botulism) follows colonization of the digestive tract by *C. botulinum* with local production of BoNT (Chia et al., 1986; McCroskey and Hatheway, 1988).

WOUND BOTULISM Like tetanus, this form of botulism results from colonization of a wound with *C. botulinum* and subsequent local production of BoNT. However, wound botulism is much less frequent than tetanus, despite the fact that the general population is not immunized against BoNT, indicating that *C. botulinum* strains have a low ability to grow in wounds compared to *C. tetani*.

ANIMAL BOTULISM Botulism is rather common among domestic and wild animals, and types C and D are involved in addition to A, B and E. Botulism of domestic animals causes economic losses, but it is also a risk factor of transmission to humans. Animal botulism can be contracted by eating the carcass or chewing on bones contaminated with BoNT, by drinking water from contaminated ponds, or by ingestion of forage poisoned by diffusion of BoNT from carcass decomposition of small animals or birds. Among fish and birds, botulism outbreak can involve millions of individuals. The disease may have the appearance of an epidemic because insect larvae that grow in the decomposing cadaver become full of BoNT, which is innocuous to insects. Since healthy birds and fish are eager for larvae, they become intoxicated and die, providing a rich anaerobic medium for the growth of *Clostridia* and for the deposition of eggs by insects. A self-perpetuating cycle is thus obtained and rapidly many individuals can die, particularly when the animal population is dense as it is in farms. Where botulism is common, animals often carry *C. botulinum* in their digestive tract, which rapidly grows in the cadavers and becomes highly toxic and rich in spores (Smith and Sugiyama, 1988; Dodds, 1993b).

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CHAPTER 1.2.22

The Enterotoxic Clostridia

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Introduction to the Enterotoxic Clostridia

The anaerobic, nutrient-rich conditions of the mammalian gastrointestinal (GI) tract provide a relatively attractive niche for many clostridia. Several of those clostridial species also produce toxins with potent activity on the GI tract, enabling them to cause human and/or veterinary enteric diseases (Table 1).

Clostridial enteric diseases vary considerably (as described in the Clinical Disease and Epidemiology sections of this chapter) but can be quite common and severe. Collectively, these infections place a major economic burden on human and veterinary medicine, draining billions of dollars per year from the economy.

This chapter focuses on the two most important enterotoxic clostridial species for human and veterinary enteric diseases, i.e., *Clostridium perfringens* and *Clostridium difficile*.

Clostridium perfringens

Introduction

Clostridium perfringens has a ubiquitous environmental distribution, including a presence in the normal GI flora of humans and domestic animals. Under favorable conditions, this bacterium can cause a wide array of enteric and histotoxic infections. This chapter focuses on the medical and veterinary enteric diseases caused by *C. perfringens*; the histotoxic infections caused by this bacterium are discussed in a separate chapter.

Historical Perspective

Clostridium perfringens has been linked to human GI disease since the mid-1940s, when McClung first noted this bacterium's ability to cause food poisoning (McDonel, 1986). This tentative association was confirmed by the classic studies of Hobbs during the 1950s (McDonel,

1986). In the late 1940s, *C. perfringens* also became associated with outbreaks of darmbrand (enteritis necroticans) in post-war Germany (Lawrence, 1997). The early 1980s saw the first linkage of *C. perfringens* with human nonfood-borne GI diseases (Borriello et al., 1984; Borriello, 1995).

The association of *C. perfringens* with veterinary enteric disease occurred even earlier, with initial reports surfacing in the 1920s and early 1930s (Songer, 1996). Appreciation of the full scope of *C. perfringens* veterinary diseases increases to this day.

Phylogeny

The genus *Clostridium* consists of nearly 150 phylogenetically heterogeneous species that do not represent a coherent taxon (Stackebrandt, 1997). Using 16S ribosomal RNA sequence analyses, 19 different clostridial species clusters have been defined (Stackebrandt, 1997; Stackebrandt et al., 1999). Along with most other pathogenic clostridial species (except *C. difficile*), *C. perfringens* belongs to phylogenetic cluster I, which is considered the core cluster of the genus *Clostridium*. Within that core cluster, *C. perfringens* belongs to the same Ia subcluster that also includes *Clostridium botulinum*.

Taxonomy

Clostridium perfringens is a Gram-positive, nonmotile, short-to-intermediate sized rod, often with a "box-car shape" (Fig. 1). This bacterium is anaerobic, as it does not grow on plates incubated under atmospheric oxygen conditions. However, *C. perfringens* is not killed quickly when exposed to air and grows after only modest reductions in environmental oxidation-reduction (E_h) conditions, perhaps because this bacterium produces relatively high amounts of superoxide dismutase (Mitchell, 2001). *Clostridium perfringens* forms spores, although sporulation is difficult to demonstrate with many isolates (particularly fresh clinical isolates).

Table 1. Major clostridial species causing human and/or veterinary enteric disease.

Species	Associated diseases	Toxins involved
C. colinum	Enterotoxemia[a] (chickens); ulcerative colitis of psittacines	?
C. difficile	Antibiotic-associated diarrhea and pseudomembranous colitis of humans; veterinary diarrheas (dogs) and necrotizing enteritis (foals)	Toxin A; toxin B; and CDT[b] (ι toxin)
C. perfringens	*C. perfringens* type A food poisoning of humans; necrotizing enteritis of humans (PigBel); antibiotic-associated and sporadic diarrhea of humans; animal enterotoxemias	*C. perfringens* enterotoxin (CPE), α toxin, β toxin, β2 toxin, ε toxin, and ι toxin[c]
C. septicum	Neutropenic enterocolitis of humans and animal enterotoxemias	α toxin
C. sordellii	Animal enterotoxemias	Lethal toxin
C. spiroforme	Animal enterotoxemias (rabbits)	ι toxin

[a]The term "enterotoxemia" is used generically in this table to indicate any serious animal infection where toxin is produced in the intestines.
[b]*Clostridium difficile* actin-specific ADP-ribosyltransferase.
[c]See Tables 2 and 3 and text for more details.

Fig. 1. Photomicrograph of Gram-stained *C. perfringens*. Note the "box car" shape. 1000× magnification.

Table 2. *Clostridium perfringens* toxin typing classification scheme.

Type[a]	Toxin produced			
	α	β	ε	ι
A	+	−	−	−
B	+	+	+	−
C	+	+	−	−
D	+	−	+	−
E	+	−	−	+

Symbols: +, produced; and −, not produced.
[a]*C. perfringens* type.

Colonies of *C. perfringens* usually reach 1–3 mm after overnight anerobic incubation on blood agar but often become much larger on longer incubation. Owing to production of hemolytic toxins (α-toxin and θ-toxin, also known as "perfringolysin O"), this bacterium typically produces a characteristic double zone of hemolysis on blood agar plates. Alpha toxin also has phospholipase C/lecithinase activity, so *C. perfringens* colonies usually exhibit lecithinase activity when grown on egg yolk agar. This bacterium causes a "stormy fermentation" of milk, hydrolyzes gelatin, ferments lactose, and reduces nitrate.

Clostridium perfringens produces a capsule whose carbohydrate composition varies between isolates. Capsular serotyping approaches have been developed to exploit this variability for distinguishing between *C. perfringens* isolates (Stringer, 1985). These capsular serotyping approaches were successfully employed during the 1950s–1980s to investigate *C. perfringens* type A food poisoning outbreaks in the United Kingdom (Stringer, 1985). However, capsular serotyping proved much less effective for investigating *C. perfringens* type A food poisoning outbreaks in the United States and Japan (where many isolates could not be typed with existing antisera), so this technique is less commonly used today.

This bacterium is a prolific toxin producer, with an intimidating arsenal consisting of at least 14 toxins (McDonel, 1986). However, only certain toxin combinations are expressed by individual isolates, forming the basis of a commonly used toxin-typing scheme for differentiating between *C. perfringens* isolates (McDonel, 1986). This toxin typing approach classifies *C. perfringens* isolates into types A–E on the basis of their production of four toxins: α, β, τ and toxins (Table 2).

This diversity of toxin expression among isolates helps explain why *C. perfringens* causes such a wide array of diseases, ranging from enteric to histotoxic infections. In that regard,

the toxin typing scheme shown in Table 2 is particularly important for understanding enteric pathogenesis, as each *C. perfringens* toxin type is associated with certain GI diseases (see Table 3).

In addition to the four typing toxins, other toxins are also clearly important for *C. perfringens* virulence. Inspection of Table 3 indicates that isolates producing *Clostridium perfringens* enterotoxin (CPE) and β2 toxin are important causes of enteric disease. Although biomedically important, only ~1–5% of all *C. perfringens* isolates, mainly belonging to type A, produce CPE (McClane, 2001a). Interestingly, nearly all type E isolates carry a defective *cpe* gene (Billington et al., 1998; see Genetics). The β2 toxin gene (*cpb*2) is more commonly distributed and can be found in any toxin type but is especially common in type E isolates (Bueschel et al., 2003).

Habitat/Ecology

The widespread environmental distribution of *Clostridium perfringens* includes a presence in soils, foods, sewage, and the GI tract of healthy or diseased humans and domestic animals. In such non-disease settings, most *C. perfringens* isolates are classified as type A and *cpe*-negative; understanding of the normal ecologic niche for the isolates causing most cases of human and veterinary enteric diseases (i.e., type A isolates producing CPE, type B, type C, type D or type E isolates) is currently only limited.

Isolation and Identification

Several selective media are commonly used for isolating *C. perfringens* from soil, foods or feces; one such selective medium is TSC (tryptose-sulfite-cycloserine) agar. *Clostridium perfringens* can be isolated by streaking a sample onto a TSC agar plate, which is then incubated anaerobically (e.g., in a Gas Pak jar) for ~18 hours. *Clostridium perfringens* colonies should appear black on this agar owing to sulfite reduction (Fig. 2A). However, perhaps 50% of all *C. perfringens* colonies appear white on TSC agar plates, and even when present initially, the black colony color often rapidly fades when examined in air. To increase the identification power of TSC agar, it is helpful to incorporate egg yolk agar into the medium. Precipitation around a colony (whether white or black) growing on TSC-containing egg yolk agar is suggestive of *C. perfringens*, which typically exhibits lecithinase activity from the phospholipase C activity of α toxin. An alternative medium commonly used for *C. perfringens* isolation is sheep blood agar containing neomycin. On this medium, most *C. perfringens* colonies are surrounded by a distinctive double zone of hemolysis due to the hemolytic activities of α and θ (perfringolysin O) toxins (Fig. 2B).

For confirmatory identification of presumptive *C. perfringens* isolates, a number of approaches can be used. Commonly used tests include evaluating whether putative isolates ferment lactose, hydrolyze gelatin, and reduce nitrate. This bacterium is also indole-negative, catalase-negative, and negative for motility in an agar deep. Gas

Table 3. Association of *C. perfringens* types with human and veterinary diseases.

Type	Major toxins	Representative diseases
A	α	Human wound infections (e.g., gas gangrene or clostridial myonecrosis), necrotic enteritis in fowls, ulcerative abomasitis and necro-hemorrhagic enteritis in cattle (not confirmed), enterotoxemia[a] in lambs and goat kids (not confirmed in goats), mild necrotizing enteritis in piglets, and enterotoxemia in South American camelides
	α, CPE	Human food poisoning, human nonfoodborne GI diseases, and veterinary diarrhea (particularly dogs, pigs, and foals)
	α, β2	Porcine GI disease, and cattle enterotoxemia (not confirmed)
B	α, β, ε	Dysentery and hemorrhagic enteritis in lambs and goat kids (not confirmed in adult goats), and biodefense concerns?
C	α, β	Human necrotizing enteritis (PigBel), necrotic enteritis in foals, hemorrhagic or necrotic enterotoxemias in neonatal pigs, calves, goats (not confirmed), foals, enterotoxemia (struck) in adult sheep, and enteritis in dogs, chickens and South American camelides
	α, β, β2	Porcine GI disease
D	α, ε	Enterotoxemia in sheep, goats (pulpy kidney disease) and cattle (not confirmed), and biodefense concerns?
E	α, τ	Rabbit, canine, bovine, ovine enterotoxemias

Abbreviations: CPE, *Clostridium perfringens* enterotoxin; and GI, gastrointestinal.
[a]The term "enterotoxemia" is used generically in this table to indicate serious GI animal infections where toxin(s) is produced in the intestines.
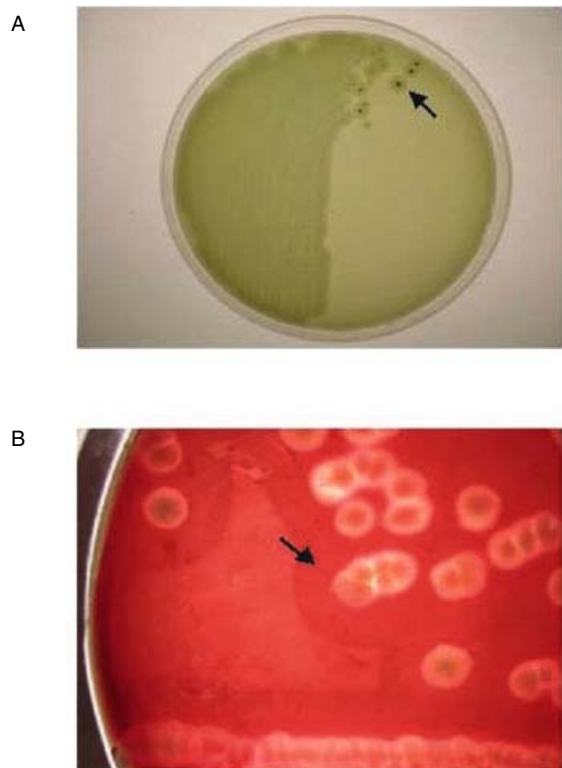
Fig. 2. Growth of *C. perfringens* on differential and selective media. A) Tryptose sulfite cycloserine (TSC) with egg yolk agar plate; note the presence of the black colonies and precipitation surrounding the colonies, and B) Sheep blood agar with neomycin; note the double zone of hemolysis surrounding the colonies.

chromatography can be used for identification of *C. perfringens*, but this approach requires more expertise, equipment, and time than biochemical identification approaches.

Preservation

Clostridium perfringens typically remains viable when stored for ~1 week at 4°C in liquid medium (such as fluid thioglycollate) or when stored frozen at –20°C for ~2 weeks. If an isolate sporulates in cooked meat medium, those cooked meat spore cultures usually remain viable for years (particularly if stored at –20°C). Other long-term (several months to many years) storage approaches include storage of vegetative cells in 50% glycerol at –80°C, or storage of lyophilized cultures (suspended in skim milk) at 4°C.

Physiology

Traditionally, *C. perfringens* has been considered a member of the saccharolytic clostridia, as it utilizes for growth a variety of carbohydrates including glucose, fructose, galactose, glycogen, lactose, maltose, mannose, raffinose, starch and sucrose. However, recent genomic analyses suggest that *C. perfringens* should also be able to ferment certain amino acids, including serine and threonine (Shimizu et al., 2002).

With respect to carbohydrate utilization for energy (Gottschalk, 1979), *C. perfringens* first converts more complex carbohydrates into simple sugars (e.g., hexoses) using degradative enzymes such as amylase. These simple sugars are then phosphorylated and taken up via phosphotransferase systems; once present inside the cell, the hexose phosphates are fermented by a classical butyric acid fermentation pathway, being first degraded via the Emden-Meyerhoff pathway to pyruvate, which is then converted to acetyl-CoA using pyruvate-ferredoxin oxidoreductase. This reaction also produces CO_2 and reduced ferredoxin, which is then oxidized (via hydrogenase) to release free hydrogen gas. The CO_2 and H_2 gases resulting from *C. perfringens* fermentations are often readily apparent as gas pockets in muscle tissue during gas gangrene. Acetyl-CoA produced by pyruvate-ferredoxin oxidoreductase is further converted by *C. perfringens* into ethanol, acetate and butyrate. The final fermentation balance (amounts formed in mol/100 mol of glucose fermented) for *C. perfringens* is 34 mol of butyrate, 60 mol of acetate, 33 mol of lactate, 176 mol of CO_2, 214 mol of H_2 and 26 mol of ethanol. The reliance of *C. perfringens* on fermentations to obtain energy can be attributed to the absence of cytochromes and tricarboxylic acid cycle (TCA) cycle enzymes in this bacterium (Shimizu et al., 2002). Despite its reliance upon fermentation for energy, *C. perfringens* has an exceptionally fast doubling time of as little as ~10 min.

Genomic analyses (Shimizu et al., 2002) indicated that, while the *Clostridium acetobutyliticum* genome encodes a complete set of genes for synthesizing amino acids, the genome of *C. perfringens* strain 13 lacks many amino acid biosynthesis genes (including the genes encoding enzymes involved in biosynthesis of arginine, aromatic amino acids, branched chain amino acids, glutamate, histidine, lysine, methionine, serine and threonine). The absence of these amino acid synthesis genes could indicate that *C. perfringens* cannot grow in environments where the amino acid supply is limiting.

Many *C. perfringens* isolates form spores, which can contribute to disease transmission of both histotoxic infections and food poisoning outbreaks. Furthermore, *C. perfringens* enterotoxin (CPE), which is responsible for the symptoms of CPE-associated human and veterinary gastrointestinal diseases, is produced only during

sporulation (McClane, 2001a). Interestingly, *C. perfringens* strain 13 genomic analyses failed to detect ~80 sporulation or germination genes present in the well-studied sporeforming bacterium *Bacillus subtilis* (Shimizu et al., 2002). These missing genes mostly encode spore coat proteins or germination-related proteins; genes encoding sporulation-associated σ factors and other stage-specific sporulation proteins are generally present in the strain 13 genome. As also previously determined for the *C. acetobutyliticum* genome (Nolling et al., 2001), strain 13 does not encode the phosphorelay system responsible for initiating sporulation in *B. subtilis* (Shimizu et al., 2002). Assuming these strain 13 findings hold true for other *C. perfringens* isolates, important differences apparently exist between the sporulation and germination processes of *C. perfringens* vs. *B. subtilis*.

Genomic analyses also revealed that *C. perfringens* strain 13 carries a large number of classical two-component regulatory systems for responding to environmental changes (Shimizu et al., 2002). Of those, only the VirR/VirS system has been extensively studied (Lyristis et al., 1994; Shimizu et al., 1994; Ba-Thein et al., 1996; Banu et al., 2000; Cheung and Rood, 2000; Ohtani et al., 2003). The VirR/VirS system plays an important role in *C. perfringens* histotoxic infections by regulating expression of α and θ toxins. As discussed later in the Toxins section of this chapter, this two-component system also probably contributes to some *C. perfringens* enteric diseases. Specifically, VirR/VirS can regulate expression of β2 toxin (Ohtani et al., 2003); whether this two-component system plays any role in regulating expression of other toxins (β, CPE, or τ toxins) involved in *C. perfringens* enteric diseases has not yet been evaluated.

Genetics

The chromosome of *C. perfringens* strain 13 is ~3.0 Mb, has a G+C content of only 28.6%, and encodes ~2.700 open reading frames (ORFs; Shimizu et al., 2002). By comparison, the ~3.7-Mb chromosome of *C. acetobutylicum* encodes ~3700 ORFs (Nolling et al., 2001). The smaller size of the *C. perfringens* strain 13 chromosome apparently renders this isolate deficient in certain metabolic pathways, such as amino acid synthesis (Shimizu et al., 2002). Whether these deficiencies are unique to strain 13 or also hold true for other *C. perfringens* isolates will soon become apparent from ongoing genome sequencing studies of two additional *C. perfringens* isolates. However, it is already clear that some *C. perfringens* isolates have much larger chromosomes (up to ~3.7 Mb) than the strain 13 chromosome (Cole and Canard, 1997).

Clostridium perfringens α toxin and perfringolysin O (a member of the streptolysin O pore-forming toxin family) are both important for histotoxic infections and are both chromosomally encoded (Canard et al., 1992; Awad et al., 1995). In contrast, most *C. perfringens* toxins linked to enteric disease (CPE, β, β2, and τ toxins) can be plasmid-encoded (Cornillot et al., 1995; Gibert et al., 1997; Collie and McClane, 1998a; Rood, 1998; Sparks et al., 2001; Miyamoto et al., 2002; Shimizu et al., 2002). With the exception of the 54-kb plasmid pCP13, which carries a β2 toxin gene in strain 13 (Shimizu et al., 2002), *C. perfringens* virulence plasmids have not yet been sequenced.

Accumulating evidence indicates that at least some virulence plasmids carrying toxin genes can horizontally transfer between *C. perfringens* isolates. For example, studies have demonstrated (Billington et al., 1998) that most, if not all, type E isolates lack clonal relationships yet carry a virulence plasmid containing an identically defective *cpe* gene (along with functional *iap* and *ibp* genes encoding τ toxin). Definitive evidence for horizontal transfer of a *C. perfringens* virulence plasmid was provided by recent mating studies clearly demonstrating transfer of a *cpe* plasmid between *C. perfringens* type A isolates (Brynestad et al., 2001). Transfer of *cpe* plasmids requires physical contact between the donor and recipients and is not affected by the presence of extracellular DNase. These observations suggest the involvement of a conjugative transfer process, although the conjugation machinery of *C. perfringens* has not yet been studied.

Transposons may also contribute to virulence gene movement in *C. perfringens*. Brynestad et al. reported that the chromosomal *cpe* gene of food poisoning isolate NCTC8239 is flanked by IS*1470* elements (Brynestad et al., 1997). From that observation, it was proposed that the NCTC8239 *cpe* gene is present on an ~6.3-kb transposon with terminal IS*1470* elements (Brynestad et al., 1997). Excision and circularization of *cpe*-containing genetic elements from the NCTC8239 chromosome was later detected; those excised elements could facilitate *cpe* gene movement (Brynestad and Granum, 1999).

Several approaches used by *C. perfringens* for regulating its virulence gene expression are also commonly employed by other Gram-positive pathogens. For example, *C. perfringens* regulates α, θ, β2 and κ toxin expression through a VirR/VirS two-component regulatory system (Lyristis et al., 1994; Shimizu et al., 1994; Ba-Thein et al., 1996; Banu et al., 2000; Cheung and Rood, 2000; Ohtani et al., 2003). This VirR-VirS regulation can be mediated through VR-RNA (Ohtani et al., 2003), a regulatory RNA (regulatory RNAs are another emerging theme for virulence gene

regulation by Gram-positive pathogens). However, *C. perfringens* also uses some relatively unusual regulatory mechanisms to regulate its virulence gene expression. For example, CPE expression during sporulation apparently involves sporulation-associated alternative σ factors (Zhao and Melville, 1998).

Phages have been identified in *C. perfringens* (Zimmer et al., 2002), but their role (if any) in virulence gene transfer is unclear at present.

Clostridium Perfringens Toxins Involved in Enteric Pathogenesis

While other *C. perfringens* toxins may also contribute to the pathogenesis of enteric disease, this section will discuss the five *C. perfringens* toxins most clearly implicated, to date, in enteric pathogenesis.

CLOSTRIDIUM PERFRINGENS ENTEROTOXIN (CPE)

Introduction In the early 1970s, Duncan's and Hauschild's groups purified CPE and demonstrated that this toxin is a 35-kDa polypeptide with heat-labile biologic activity (McDonel, 1986). CPE was later shown to lack homology with other known proteins, except for some limited homology (of unknown significance) with several nonneurotoxic proteins made by *C. botulinum* (Melville et al., 1997).

Genetics The gene *cpe* encoding this enterotoxin can be present on either the chromosome (where it may be part of Tn*5565*, a putative 6.5-kb transposon with terminal IS*1470* repeats) or on a large plasmid (Cornillot et al., 1995; Brynestad et al., 1997; Collie and McClane, 1998a; Brynestad and Granum, 1999; Sparks et al., 2001; Miyamoto et al., 2002). No single isolate has yet been found that carries both chromosomal and plasmid-borne *cpe* genes. The *cpe* plasmid of isolate F4969 can transfer via conjugation (Brynestad et al., 2001).

Interestingly, CPE-positive type A isolates causing food poisoning usually carry a chromosomal *cpe* gene, while the CPE-positive type A isolates causing nonfoodborne human GI diseases typically carry a plasmid *cpe* gene (Cornillot et al., 1995; Collie and McClane, 1998a; Collie et al., 1998b; Sparks et al., 2001; Wen et al., 2003). Possible reasons for these strong *cpe* genotype disease associations are now emerging. For example, both cells and spores of chromosomal *cpe* isolates exhibit much stronger heat resistance than the cells and spores of plasmid *cpe* isolates (Sarker et al., 2000). Thus, it is more likely for those heat-resistant, chromosomal *cpe* isolates to cause a food poisoning typically

spread via cooked meat products. Furthermore, the demonstrated ability of at least some *cpe* plasmids to transfer between *C. perfringens* type A isolates (Brynestad et al., 2001) might help initiate the CPE-associated nonfoodborne diseases. Unlike *C. perfringens* type A food poisoning, which is thought to result from ingestion of large numbers of *C. perfringens* vegetative cells present in grossly-contaminated foods (McClane, 2001a), CPE-associated nonfoodborne diseases appear to be caused by ingestion of only a low infecting dose of *C. perfringens* cells (Borriello et al., 1985c; Borriello, 1995). Thus, it should help establish disease for those few infecting isolates to transfer their *cpe* plasmid to normal flora, *cpe*-negative *C. perfringens* isolates. This putative in vivo transfer of the *cpe* plasmid to normal flora strains, which are presumably under selective pressure to colonize the GI tract, could also help explain why symptoms of CPE-associated nonfoodborne human GI diseases persist longer than those of *C. perfringens* type A food poisoning, which involves only exogenous strains probably not well-suited for intestinal colonization.

Significant organizational differences exist between the arrangements of plasmid vs. chromosomal *cpe* loci in type A isolates (Miyamoto et al., 2002; Fig. 3). These organizational differences do not affect the regulation of CPE expression, as all *cpe*-positive isolates express the enterotoxin only during sporulation (Collie et al., 1998b). The sporulation-associated nature of CPE expression is mediated at the transcriptional level (Melville et al., 1994; Czeczulin et al., 1996; Zhao and Melville, 1998) and apparently involves three highly conserved promoters located immediately upstream of the *cpe* ORF (Zhao and Melville, 1998). These *cpe* transcriptional start sites share homology with SigE- and SigK-dependent promoters, which appear important since SigE and SigK 1) are encoded by the *C. perfringens* chromosome (Shimizu et al., 2002), and 2) perform gene regulatory functions in sporulating *B. subtilis* mother cells (Zhao and Melville, 1998).

Whether carrying a chromosomal or plasmid *cpe* gene, isolates often produce extremely large amounts of the enterotoxin. CPE can represent >15% of the total protein inside a sporulating cell (Czeczulin et al., 1993; Collie et al., 1998b). The abundant expression of CPE by these sporulating cells could result, at least in part, from message stability, as older studies suggest that *cpe* mRNA has an exceptionally stable half-life of ~45 min (Labbe and Duncan, 1977). This unusual message stability could be mediated, at least in part, by the presence of a stem-loop structure, followed by an oligo T tract, downstream of the *cpe* termination codon (Czeczulin

I. Type A Plasmid *cpe* Isolates

A F4969

dcm
ORF 1–1017

IS *1469*
ORF 2067–2619

cpe
ORF 3874–4830

IS *1470-like*
ORF 6075–7182

B F4013

dcm

IS *1469*

cpe

IS *1151-like*
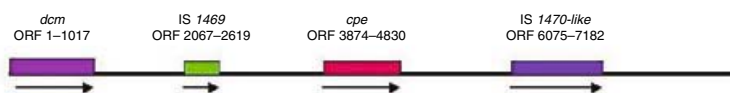
II. Type A Chromosomal *cpe* Isolates

NCTC8239

uapC   IS *1470*   IS *1469*   *cpe*   IS *1470*   nadC

500 base

Fig. 3. Genetic organization of the *cpe* locus in *C. perfringens* type A isolates. I. The plasmid *cpe* locus in type A isolates carrying either downstream IS*1470*-like sequences (A) or downstream IS*1151* sequences (B). II. The chromosomal *cpe* locus of type A isolates. Modified with permission from Miyamoto et al. (2002).

High CPE dose   Low CPE dose

Ions   Ions (More Influx)   Ions (Less Influx)

Oncosis   Apoptosis

Cell damage and DNA fragmentation   Tight junction damage

Paracellular permeability change

Histopathologic damage and diarrhea

Fig. 4. Comprehensive model of *Clostridium perfringens* enterotoxin (CPE) action. See text for detailed explanation.

et al., 1993); that downstream loop also likely functions as a ρ-independent transcriptional terminator (Czeczulin et al., 1993).

Mechanism of Action CPE has lethal (LD_{50} [50% lethal dose] via i.v. route = 1 µg/mouse), cytotoxic, and enterotoxic activities, as well as the ability to cause intestinal histopathologic damage. A comprehensive model depicting the current understanding of CPE action is shown in Figure 4 and will now be briefly explained.

At pathophysiologic concentrations, CPE first binds to protein receptors (McClane and McDonel, 1981). Some recent experiments

(Katahira et al., 1997a; Katahira et al., 1997b; Fujita et al., 2000) suggest the CPE receptor(s) might include certain claudins, which are a large (>20 members) family of ~22-kDa proteins that serve structurally important roles in epithelial tight junctions (TJs). In those recent studies, a series of rat fibroblasts, which do not normally express any claudins or naturally bind or respond to CPE, were transfected to express a single claudin. Expression of claudin-3, -4, -6, -7, -8, or -14 (but not claudins-1, -2, -5 or -10) was sufficient to convert those fibroblast transfectants to CPE-sensitivity, suggesting those particular claudins could be CPE receptors. However, this conclu-

sion remains tentative since direct interactions between CPE and claudins have not yet been demonstrated in enterocytes or cultured enterocyte-like cells (such as human Caco-2 cells). This point is important since co-immunoprecipitation studies determined that an ~50-kDa protein interacts with CPE soon after binding to Caco-2 cells (Wieckowski et al., 1994). The identity of that ~50-kDa protein is not yet clear, but those co-immunoprecipitation results could indicate 1) the ~50-kDa protein is the actual CPE receptor on natural CPE target cells, 2) there are multiple, independent CPE receptors on those cells, 3) the ~50-kDa protein is a claudin aggregate, or 4) claudins and the ~50-kDa protein serve together as co-receptors for CPE binding.

Upon binding to naturally sensitive cells, such as Caco-2 cells, CPE becomes localized in a small (~90-kDa) complex (Fig. 5). Formation of this small complex appears important for CPE action, as this CPE species is formed by all sensitive cells (Wieckowski et al., 1994). However, formation of the small complex is not sufficient for obtaining toxicity, as several CPE point mutants form the small complex, yet are nontoxic (Kokai-Kun et al., 1999). Furthermore, when native CPE is applied to sensitive cells at

Fig. 5. *Clostridium perfringens* enterotoxin (CPE) complex formation in rabbit intestinal brush border membranes (BBMs). Right panel: ^{125}I-CPE treatment of BBMs at 4°C in the presence (+) or absence (–) of excess unlabeled CPE. As unbound toxin was washed away, the BBMs were extracted with Triton X-100 and analyzed by nondenaturing gel electrophoresis. SC indicates migration of the small (90-kDa) CPE complex formed under this condition. Left panel: The same experiment as shown in the right panel, except ^{125}I-CPE treatment of BBMs was performed at room temperature (RT; not shown) or 37°C instead of 4°C. Note the presence of the additional higher M_r band that contains the larger CPE complexes (LC). If the BBMs treated at 4°C as in the right panel are washed and shifted to 37°C, some of the radioactivity present in the small complex now migrates with the larger complexes (not shown). For comparison, the migration of free CPE (no BBMs present) in this gel system is shown (CPE). From Wieckowski et al. (1994) with permission.

4°C, the bound toxin localizes only in the small complex, yet those CPE-treated cells remain unaffected (McClane and Wnek, 1990; Kokai-Kun et al., 1999).

If Caco-2 cells are treated with CPE at 4°C, washed to remove unbound CPE, and then shifted to 37°C, they rapidly develop cytotoxic responses (McClane and Wnek, 1990). Concurrently, with the onset of cytotoxicity in those cells, some bound CPE in small complex becomes localized in much larger plasma membrane complexes (Fig. 6). This finding indicates small complex formation is a precursor for formation of the larger CPE complexes and that those larger complexes are responsible for cytotoxicity. The involvement of the larger CPE-containing complexes in CPE cytotoxicity receives further support from 1) the identification of several nontoxic CPE point mutants that can bind and form the small complex but not the larger CPE complexes (Kokai-Kun et al., 1999), and 2) the identification (Kokai-Kun and McClane, 1997a) of several CPE deletion fragments that show, relative to native CPE, enhanced cytotoxic activity and better large complex-forming ability (see Structure/Function section).

The larger CPE complexes were recently resolved into two main species of ~155 kDa and ~200 kDa (Fig. 6). The TJ protein occludin is also present in the ~200-kDa complex (Fig. 6). A follow-up study (Singh et al., 2001) determined that 1) ~155-kDa CPE complex formation is sufficient for inducing cytotoxicity and 2) formation of the ~200-kDa complex removes occludin from TJs, with the TJ protein then internalized into the cytoplasm of Caco-2 cells. This occludin internalization effect, perhaps coupled with similar internalization of the claudins (another TJ component), could explain (McClane, 2000) CPE-induced damage to TJ structure and function (e.g., paracellular permeability alterations). This same study (Singh et al., 2001) also demonstrated the sensitivity of polarized Caco-2 cells to CPE-treatment of their apical surface, which is the cell surface initially exposed to CPE. Interestingly, Caco-2 cells were found to be even more sensitive when CPE is applied to their basolateral surface (Fig. 7).

Increasing evidence suggests that, when localized in the ~155-kDa complex, CPE forms pores that cause the plasma membrane permeability alterations responsible for cytotoxicity (Kokai-Kun and McClane, 1996; Wieckowski et al., 1998; Hardy et al., 1999). It is now clear that cell death pathways triggered by membrane permeability alterations are CPE dose-dependent (Chakrabarti et al., 2003). At low CPE concentrations, where plasma membrane permeability alterations develop more slowly, a classical caspase 3-mediated apoptosis develops. However, at

Kinetics of Large Complex Formation at 37°C

Fig. 6. Larger *Clostridium perfringens* enterotoxin (CPE) complex formation in CaCo-2 cells treated with CPE at 37°C. After the specified incubation times, CPE-treated cell suspensions were centrifuged, extracted with sodium dodecylsulfate (SDS), electrophoresed on 4% acrylamide gels containing SDS, and then Western immunoblotted with antibodies specific for either CPE (left panel) or occludin (right panel). Red and orange arrows show the migration of the ~200- and ~155-kDa CPE complexes, respectively. The green arrow shows migration of another CPE complex of ~135 kDa that is sometimes discernible in this experiment. From Singh et al. (2000) with permission.

higher CPE concentrations, more rapid development of membrane permeability changes occurs and caspase 3-independent oncosis is triggered.

CPE-induced enterocyte death via either apoptosis or oncosis produces histopathologic damage in vivo (McDonel, 1986). Tissue damage appears responsible for initiating intestinal fluid and electrolyte loss (McDonel, 1986; Sherman et al., 1994). CPE effects on paracellular permeability from TJ protein internalization may also contribute to intestinal fluid and electrolyte loss (McClane, 2000). Inflammation could also contribute to intestinal fluid and electrolyte losses, particularly when the high CPE doses that induce the proinflammatory process of oncosis are present (Chakrabarti et al., 2003).

CPE Structure/Function Relationships The 319-amino acid CPE polypeptide resembles many bacterial toxins by segregating its toxicity and binding domains (Fig. 8). Receptor binding activity resides in the extreme C-terminal portion of the enterotoxin (Hanna et al., 1989; Hanna et al., 1991; Hanna et al., 1992; Kokai-Kun and McClane, 1997a). Sequences necessary for toxicity are located in the N-terminal half of CPE (Kokai-Kun and McClane, 1996; Kokai-Kun and

McClane, 1997a); those sequences are required for formation of the ~155-kDa and ~200-kDa large complexes.

Interestingly, removing the first ~45 N-terminal amino acids from the native enterotoxin activates toxicity (Kokai-Kun and McClane, 1997a). This activation is not attributable to removal of a signal peptide, since CPE is not a secreted protein (Czeczulin et al., 1993). Intestinal proteases, such as trypsin and chymotrypsin, can activate the enterotoxin in vitro (Granum et al., 1981; Granum and Richardson, 1991), so similar proteolytic activation may occur in the intestines during GI disease.

A neutralizing linear epitope is present in the extreme C-terminal receptor-binding region of CPE (Hanna et al., 1992). A synthetic peptide corresponding to the C-terminal CPE sequences containing the neutralizing epitope induces formation of CPE-neutralizing antibodies (Mietzner et al., 1992). This observation supports the possible use of nontoxic C-terminal CPE sequences for development of a CPE vaccine.

CPE-associated Diseases CPE is important for the pathogenesis of *C. perfringens* type A food poisoning, CPE-associated nonfoodborne human GI diseases, and certain veterinary GI

CPE Cytotoxicity For Transwell Cultures of CaCo-2 and Vero Cells

CaCo-2 Cells   Vero Cells
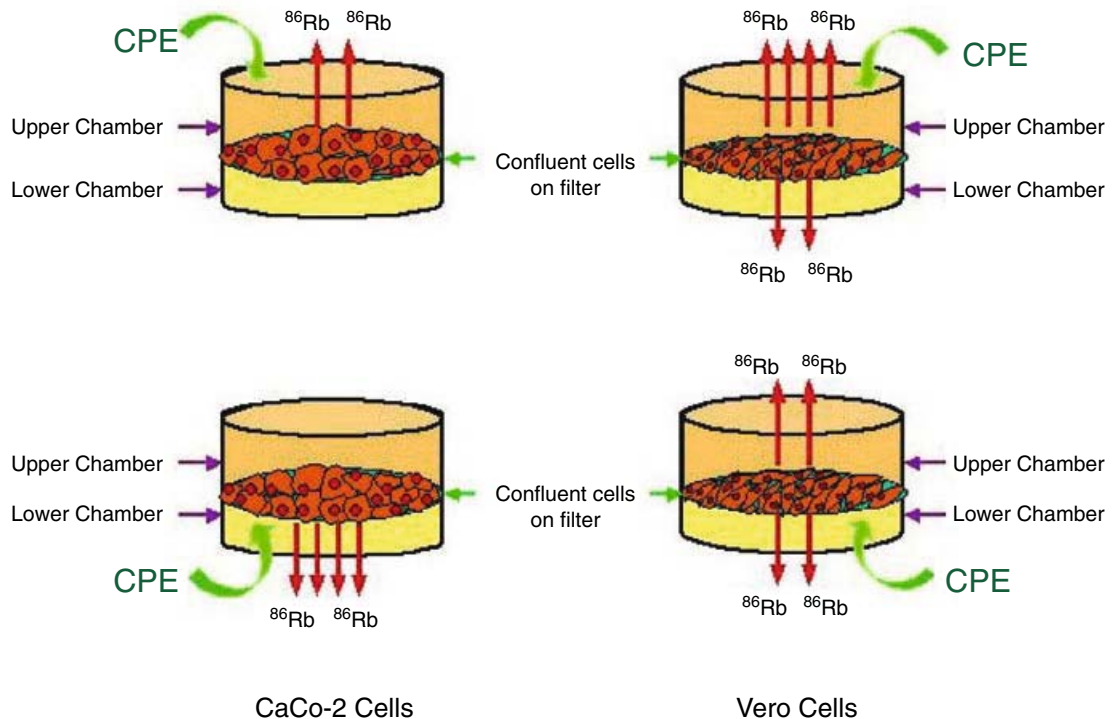
Fig. 7. Cytotoxic effects of CPE on Transwella™ cultures of CaCo-2 cells and Vero cells. Cultures were radiolabeled with [86]RbCl for 2 h. After washing to remove unincorporated radiolabel, the cultures were treated with *Clostridium perfringens* enterotoxin (CPE), which was added (as indicated) to the top or bottom Transwella™ chamber. After 15 min incubation, culture supernatants were removed from each Transwella™ chamber and counted for radioactivity. The number of arrows shown corresponds to the relative level of ^{86}Rb release into that chamber. Note that Vero cells, which lack tight junctions, release radiolabel into both chambers. In contrast, CaCo-2 cells, which are polarized enterocyte-like cells forming tight junctions, release radiolabel only into the same chamber containing CPE. Also note that CaCo-2 cells are much more sensitive to CPE when treated on their basal surface. From McClane (2001b) with permission.

diseases (see Epidemiology and Disease sections for details).

Beta Toxin

Introduction Uncertainty regarding the biochemical properties (molecular mass, sequence, etc.) of *C. perfringens* β toxin was resolved when the gene (*cpb*) encoding this toxin was cloned and sequenced in the early 1990s (Hunter et al., 1993). These studies revealed that the *cpb* gene encodes a protein of 336 amino acids, including a 27-amino acid signal peptide. This processing leaves mature β toxin with a molecular mass of ~34.9 kDa.

Genetics Initial studies detected minor sequence differences between the *cpb* ORF sequence in a type B isolate vs. a type C isolate (Hunter et al., 1993; Steinsthorsdottir et al., 1995); whether
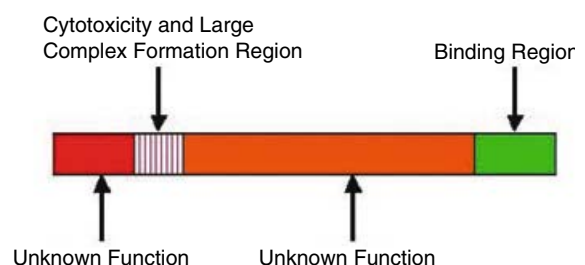
Fig. 8. *Clostridium perfringens* enterotoxin (CPE) structure/function relationship map. CPE regions important for the formation of large complexes/cytotoxicity and receptor binding are shown on this map.

these sequence differences hold true for other type B and C isolates is not yet clear. No studies have yet investigated the *cpb* promoter(s) or *cpb* regulation.

The *cpb* gene often, if not always, resides on large plasmids (Duncan et al., 1978), which have not yet received much study.

Mechanism of Action Beta toxin has lethal (LD_{50} via i.v. route = ~100 ng/mouse), dermonecrotic, and pressor activities (Leary and Titball, 1997).

Recent studies demonstrated that, reminiscent of CPE, β toxin forms at least two species of large complexes in sensitive HL60 cells (Nagahama et al., 2003). These complexes of ~191 kDa (presumably consisting of a β toxin hexamer) and ~228 kDa (presumably consisting of a β toxin heptamer) localize in lipid rafts. Formation of the ~228-kDa complex appears responsible for inducing membrane permeability alterations in HL60 cells. Whether similar membrane permeability effects are responsible for the in vivo effects of β toxin is not yet clear.

Structure/Function Relationships The amino acid sequence of β toxin shares 28% similarity with *Staphylococcus aureus* β toxin, which also alters plasma membrane permeability by forming heptameric complexes corresponding to plasma membrane pores (Hunter et al., 1993; Steinsthorsdottir et al., 1995).

The biologic activity of β toxin is susceptible to trypsin, helping to explain why people with diets rich in sweet potatoes, which contain a trypsin inhibitor, or suffering from pancreatic diseases are particularly susceptible to β toxin-mediated enteritis necroticans (Lawrence, 1997).

Associated Diseases Beta toxin contributes to the pathogenesis of necrotizing enteritis in humans, as well as veterinary necrotic enteritis and enterotoxemias (see Epidemiology and Disease sections).

Beta-2 Toxin

Introduction A 28-kDa toxic protein purified from type C strain CWC245 in the mid-1980s was originally identified as β toxin (Jolivet-Reynaud et al., 1986). However, subsequent cloning and sequencing studies indicated that the β toxin gene *cpb* actually encodes an ~34-kDa protein (Hunter et al., 1993; Steinsthorsdottir et al., 1995). This discrepancy was resolved in the mid-1990s when Popoff's group cloned and sequenced the gene encoding the 28-kDa protein produced by strain CWC245 (Gibert et al., 1997). These results unambiguously demonstrated that the 28-kDa protein is not β toxin but is instead a distinct new protein named β2 toxin.

Genetics The β2 toxin gene (*cpb2*) encodes a 265-amino acid protein, which includes an ~30-amino acid signal peptide for secretion (Gibert et al., 1997). The β2 toxin sequence shares no homology with β toxin or other known proteins.

The *cpb* 2 gene, which can be found in all *C. perfringens* types, resides on a large plasmid in most, if not all, isolates (Gibert et al., 1997). One *cpb2*-carrying plasmid, pCP13, has been sequenced; pCP13 does not encode any other toxins (Shimizu et al., 2002).

Studies are beginning to investigate the regulation of *cpb2* expression (Gibert et al., 1997; Ohtani et al., 2003). Promoter sequences reside immediately upstream of the *cpb2* start codon. A hairpin loop, which could be a ρ-independent transcriptional terminator, lies immediately downstream of the *cpb2* termination codon. The *cpb2* gene is maximally transcribed during the mid-exponential growth phase. Expression of β2 toxin is regulated via the VirR/VirS two-component regulatory system, which also regulates several other *C. perfringens* toxins (e.g., β toxin and perfringolysin O). VirR/VirS regulates expression of β2 toxin through a regulatory RNA named "VR-RNA."

Mechanism of Action Beta-2 toxin is lethal for mice (LD_{50} via i.v. route = ~3 μg/mouse) and cytotoxic for Chinese hamster ovary (CHO) and I407 cells (Gibert et al., 1997). It also has enterotoxic properties, causing fluid accumulation in guinea pig ileal loops, where it also induces substantial necrosis (Gibert et al., 1997). The molecular action of this toxin is not yet clear.

Structure/Function Relationships This issue has not yet been explored.

Associated Diseases Beta-2 toxin has been linked to several veterinary GI diseases, most notably porcine enteritis (see Epidemiology and Diseases sections for further details).

Epsilon Toxin

Introduction As discussed in the Epidemiology/Disease sections of this chapter, toxin has traditionally been viewed as only a veterinary concern. However, as the third most potent clostridial toxin (after botulinum and tetanus toxins), toxin is now a feared bioterrorism agent and thus listed as a class B select agent.

Genetics The gene (*etx*) encoding toxin produces, after removal of a 13-amino acid signal peptide during secretion, a 311-amino acid prototoxin (Hunter et al., 1992; Payne and Oyston, 1997). This prototoxin is relatively inactive until proteolytically processed (see Structure/Function Relationships, below). It has been suggested that promoter differences exist between the *etx*

genes of type B vs. D isolates (Hunter et al., 1992; Payne and Oyston, 1997), but this has not yet been proven.

In most or all type B and D isolates, the *etx* gene is present on large plasmids (Betancor et al., 1999). These plasmids have not yet been studied in detail, but in at least one type D isolate, the same plasmid can carry both the *etx* and *cpe* genes (Dupuy et al., 1997).

Mechanism of Action Epsilon toxin is highly lethal (LD_{50} via i.v. route = ~75 ng/mice), neurotoxic, enterotoxic, and cytotoxic (for Madin-Darby canine kidney [MDCK] cells). It can also induce intestinal histopathologic damage and edema in many organs (Payne and Oyston, 1997).

After being proteolytically activated in the gut, toxin affects the intestines, where it causes luminal fluid accumulation (Fernandez Miyakawa and Uzal, 2003). Epsilon toxin also induces histopathologic damage in the colon, which could assist the absorption of this toxin into the blood (Fernandez Miyakawa and Uzal, 2003). Once present in the blood, toxin circulates to all organs, resulting in numerous pathophysiologic effects, such as kidney necrosis, elevation of blood pressure, and increased vascular permeability (Payne and Oyston, 1997). The toxin also crosses the blood-brain barrier, where it causes edema and neuron damage that results in neurologic disorders, such as convulsions (see Diseases for more detail).

Molecular details of toxin action are now under intense study. Increasing evidence (Miyata et al., 2002; Petit et al., 2003) indicates that, at least in the sensitive MDCK cell culture model, toxin binds to an unidentified receptor and then forms a heptamer (Fig. 9). This toxin complex is present in lipid rafts and acts as a pore-like structure to alter plasma membrane permeability. Details of how this toxin effect on plasma membranes induces neurotoxicity, etc. remain unknown.

Structure/Function Relationships Studies (Miyata et al., 2001) have shown that the relatively inactive 311-amino acid prototoxin is converted to active toxin by either intestinal proteases (such as trypsin) or by *C. perfringens* proteases (such as λ toxin). These proteases remove 13 N-terminal and 22 C-terminal amino acids from the prototoxin; removal of the C-terminal amino acids is essential for heptamerization and thus (presumably) biologic activity.

Associated Diseases Epsilon toxin plays an important role in veterinary enterotoxemias caused by type B and D isolates of *C. perfringens* (see the Epidemiology and Diseases sections). As mentioned, the extreme potency of this toxin also makes it a bioterrorism concern, warranting its listing as a class B select agent.

IOTA TOXIN

Introduction Clostridium perfringens τ toxin is a member of the binary toxin family, which also includes *C. spiroforme* τ toxin, *C. difficile* CDT toxin, *C. botulinum* C2 toxin, and *B. anthracis*

Fig. 9. Formation of a large sodium dodecylsulfate (SDS)-resistant complex by recombinant ε-toxin species in synaptosomal membranes. ^{125}I-labeled recombinant ε-toxin species were bound to synaptosomal membranes, which were then washed and extracted with SDS. These SDS extracts were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), followed by autoradiography. Lane 1, recombinant ε-prototoxin; lane 2, recombinant ε-prototoxin lacking 13 N-terminal amino acids naturally removable by proteases; lane 3, recombinant ε-prototoxin lacking 22 C-terminal amino acids naturally removable by proteases; lane 4, recombinant ε-toxin lacking both the 13 N-terminal and 22 C-terminal amino acids naturally removable by proteases. Note the heptamerization in lanes 3 and 4. These recombinant ε-toxin species in lanes 3 and 4 also had LD_{50} values nearly 100-fold lower than the species shown in lanes 1 and 2. From Miyata et al. (2001) with permission.

Type E Strain
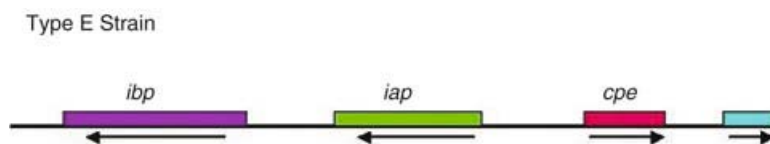
Fig. 10. Organization of the plasmid-borne τ toxin gene locus in type E isolates. Note the presence of an IS*1151* sequence and a defective *cpe* gene immediately upstream of the *iap* and *ibp* genes. From Billington et al. (1998) with permission.

anthrax toxin (Carman et al., 1997b). These binary toxins all consist of binding and toxic polypeptides that only associate after the binding component becomes localized on the mammalian cell surface.

Genetics Iota toxin is encoded by two genes (Carman et al., 1997b). Both *iap* and *ibp* encode proteins with three domains: an N-terminal signal peptide, a propeptide, and the mature binding protein (see Structure/Function section for further description).

The *iap* and *ibp* genes are typically present together on the same large plasmid, where they lie in close proximity (Fig. 10). The close proximity of the *iap* and *ibp* genes allows them to form a bifunctional operon (Carman et al., 1997b). The transcriptional start site for *iap* lies ~135 bp upstream of the start codon. The *ibp* gene is also transcribed from the *iap* promoter, although inefficient transcription can occur from a promoter directly upstream of the *ibp* start codon.

Interestingly, the same plasmid carrying the *iap* and *ibp* genes typically has a defective *cpe* gene located immediately upstream of the τ toxin genes (Billington et al., 1998). It has been proposed that this plasmid resulted from the integration of a genetic element carrying the τ toxin gene near what was a functional, plasmid-borne *cpe* gene, with this insertion event inactivating the *cpe* promoter. Possible transfer of genetic elements carrying τ toxin genes is supported by the presence of binary toxin genes in several species of sporeforming bacteria. As type E isolates apparently share similar plasmids but lack chromosomal clonality, the plasmid carrying functional τ toxin genes and a defective *cpe* gene may have horizontally transferred between *C. perfringens* isolates.

Mechanism of Action Iota toxin has dermonecrotic, lethal (LD_{50} via i.v. route = ~100 ng/mouse), cytotoxic, and enterotoxic properties (Carman et al., 1997b). The toxin can also induce intestinal histopathologic damage.

The molecular action of τ toxin involves proteolytic removal of a propeptide from both τ a and τ b (Gibert et al., 2000). The activated τ b binds to still unidentified protein receptors on mammalian cells (Carman et al., 1997b). The activated τ a component then binds to the cell-associated τ b component. The resultant toxin complex is internalized via endocytosis. The τ b component then probably mediates entrance of the τ a component into the cytoplasm, where it ADP-ribosylates actin at the Arg-177 residue. How that effect translates into intestinal pathology is not yet clear.

Structure/Function Relationships Iota toxin has become very well-studied at the structural level (Sakurai et al., 2003). As mentioned, it now appears that the τ a component must be proteolytically activated (Gibert et al., 2000). This can be achieved by either *C. perfringens* λ toxin or chymotrypsin, which removes 9–13 amino acids from the N-terminus of the τ a prototoxin. Sequence comparisons indicated that the mature τ a component contains all three conserved regions of classical ADP-ribosylating bacterial toxins 1) the aromatic residue-arginine/histidine (R/H) motif involved in nicotinamide adenine dinucleotide (NAD) binding, which includes Phe-349 and Arg-295/296 of τ a, 2) the hydrophobic residue-serine-threonine-serine (S-T-S) sequence that forms the floor of the catalytic cavity, which includes Ser-338, Ser-340 and Thr-339 of τ a, and 3) the glutamate-aspartate-glutamate (E-X-E) motif extending towards the catalytic cavity, which involves Glu-378 and Glu-380 of τ a. Site-directed mutagenesis studies have now confirmed the importance of most of these amino acid residues for the ADP-ribosylating activity of τ a (Sakurai et al., 2003). The crystal structure of τ a complexed with nicotinamide adenine dinucleotide phosphate (NADPH) was recently solved at 1.8Å resolution (Fig. 11). This structure indicates τ a is a two-domain protein consisting of a N-terminal domain (amino acids 1–210) and a C-terminal domain (amino acids 211–413). Both domains have a β-sandwich core surrounded by α helices. NADH or NADPH binds to a cavity located in the C-terminal domain. The aromatic residue-R/H, S-T-S and E-X-E regions important for ADP-ribosylating activity are also present in a cavity present in the C-terminal domain.
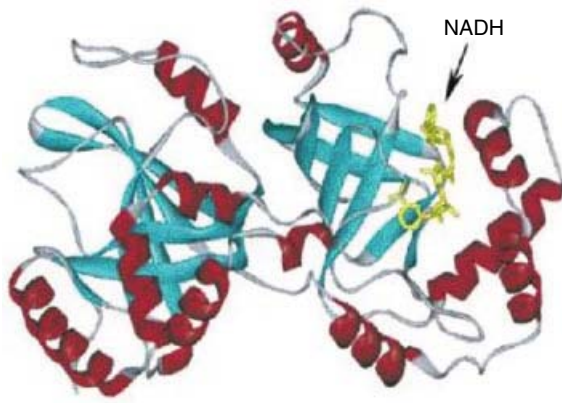
Fig. 11. Structure of the τ a component of τ toxin. Secondary structure elements are shown in red (α helices), light blue (β strands) and gray (loops). Note the location of the NADH binding site in the C-domain. From Sakurai et al. (2003) with permission.

Studies of the τ b component indicate that the 39 N-terminal amino acids encoded by the *ibp* ORF are a signal peptide that is removed during secretion (Carman et al., 1997b). The resultant prototoxin includes a 171 propeptide that is then removed by bacterial and intestinal proteases to form the mature τ b binding component of 664-amino acid residues.

Associated Diseases Iota toxin has been linked to several veterinary GI diseases caused by type E isolates of *C. perfringens* (see Diseases and Epidemiology sections).

Epidemiology: *C. perfringens* Gastrointestinal Diseases of Humans

CLOSTRIDIUM PERFRINGENS TYPE A FOOD POISONING CPE-positive *C. perfringens* type A isolates are the second and third most common cause of foodborne disease in the United Kingdom and United States, respectively (Bean et al., 1996; Adak et al., 2002). Recent, likely conservative, United States Centers for Disease Control and Prevention (CDC) estimates indicate that ~250,000 cases of this illness occur annually in the United States (Mead et al., 1999). *Clostridium perfringens* type A food poisoning is often mild, but can result in fatalities, particularly in the elderly or debilitated persons. For example, this food poisoning kills ~7 persons/year in the United States (Mead et al., 1999) and ~50–100 persons/year in the United Kingdom (Adak et al., 2002).

Two unusual epidemiologic features of *C. perfringens* type A food poisoning outbreaks are their unusually large size and their association with institutionalized settings (McClane, 2001a).

These epidemiologic patterns are probably attributable to two factors: 1) institutions often prepare foods in advance and then must hold that food for later serving, allowing the rapid growth of *C. perfringens* in any temperature-abused food (recall that the doubling time of this bacterium can be as little as ~10 min), and 2) public health officials usually confirm the identity of *C. perfringens* type A food poisoning cases only when large numbers of people become ill in a single outbreak.

The most commonly involved food vehicles in *C. perfringens* type A food poisoning outbreaks are meats and poultry (McClane, 2001a). Stews, gravies and Mexican foods are also important for transmission of this illness. Temperature abuse during storage or holding of foods is a contributing factor to nearly 100% of all *C. perfringens* type A food poisoning outbreaks, while incomplete cooking of foods or use of contaminated food preparation equipment are involved in ~30% and 15%, respectively, of all outbreaks of this disease. The importance of temperature abuse during cooking and holding of foods in the development of *C. perfringens* type A food poisoning is not surprising given the exceptional heat resistance noted for spores and vegetative cells of food poisoning isolates (Sarker et al., 2000).

The best way to reduce the frequency of *C. perfringens* type A food poisoning outbreaks is, first, to thoroughly cook foods. This is particularly true of the large roasts and turkeys that are often vehicles for this food poisoning and where it is more difficult to reach the high internal temperatures necessary to kill *C. perfringens* cells and spores. After cooking, it is then essential to cool foods rapidly and then store those foods under nonpermissive growth conditions for *C. perfringens*, e.g., at 4°C.

CPE expression appears to be both required and sufficient for the pathogenesis of *C. perfringens* type A food poisoning. Specific inactivation of the *cpe* gene eliminates the ability of a human food poisoning isolate to cause histopathologic damage or fluid accumulation in rabbit ileal loops (Fig. 12). Enteric virulence can be fully restored by complementing that *cpe* knock-out mutant with a wild-type *cpe* gene (Fig. 12), i.e., this human food poisoning isolate must express CPE to cause intestinal effects in this animal model. Additionally, human volunteer feeding studies conducted in the 1970s demonstrated that ingestion of purified CPE is sufficient to reproduce the cramping and diarrheic symptoms of this food poisoning (Skjelkvale and Uemura, 1977).

CPE-ASSOCIATED NONFOODBORNE GI DISEASES The epidemiology of CPE-associated nonfood-

Histologic Damage Induced By CPE-Positive
Clostridium perfringens Strain SM101

WILD-TYPE, FTG   WILD-TYPE, DS

cpe KNOCK-OUT, DS   COMPLEMENTING, DS
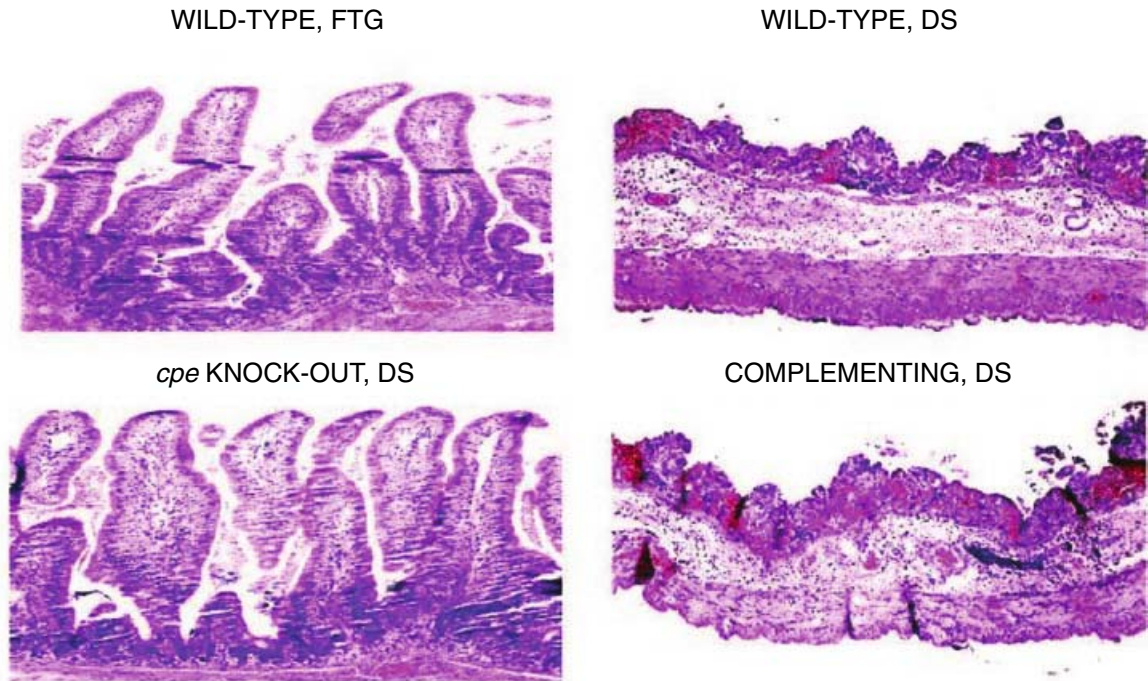
Fig. 12. Histopathologic damage induced by culture lysates of *C. perfringens* type A food poisoning isolate SM101 and derivatives. Tissue specimens shown were collected from rabbit ileal loops treated with either concentrated lysates from vegetative (fluid thioglycolate [FTG] medium) or sporulating (Duncan and Strong [DS] medium) cultures of wild-type *C. perfringens* isolate SM101 (a CPE-positive, transformable strain derived from food poisoning isolate NCTC8798; Zhao and Melville, 1998), the isogenic *cpe* knock-out mutant MRS101, or the *cpe*-complementing strain MRS101(pJRC200). Note extensive epithelial desquamation and severe villi shortening are present in loop samples treated with DS lysates of SM101 or MRS101 (pJRC200) but not in loops treated with FTG lysates of SM101 or DS lysates of MRS101. Similarly, no tissue damage was apparent (not shown) in loops treated with concentrated FTG lysates of MRS101 or MRS101(pJRC200). Ileal loop fluid accumulation was only present in those loop samples showing histopathologic damage. From Sarker et al. (1999) with permission.

borne GI diseases is still incompletely understood. Some evidence suggests these illnesses result from ingestion or inhalation of environmental *C. perfringens* isolates (Borriello et al., 1985c). These illnesses are more prevalent in the elderly and in people taking antibiotics. They can often occur in nosocomial settings, where they may be transmitted person-to-person.

ENTERITIS NECROTICANS Type C isolates of *C. perfringens* cause enteritis necroticans, a potentially lethal human enteric infection (Johnson and Gerding, 1997; Lawrence, 1997). This illness was first noted in malnourished Germans at the end of World War II, where it was named "darmbrand" ("fire bowels"). The most thorough epidemiologic studies of enteritis necroticans to date have been conducted in the

highlands of Papua New Guinea (PNG), where this illness is known as "PigBel." This illness is also endemic in other developing areas of Southeast Asia. Enteritis necroticans from type *C. perfringens* occasionally occurs in developed countries, where it often involves people with pancreatic or intestinal disease.

In PNG, about 70% of PigBel cases occur in children, with a peak incidence in 4-year-olds. Infants do not typically become ill with PigBel, perhaps because of maternal immunity. Interestingly, the peak ages affected by darmbrand were infants and the elderly.

As described in the Clinical Illness section of this chapter, sanitation, malnourishment and/or underlying pancreatic and intestinal disease play important contributing roles in the development of enteritis necroticans.

In 1980, a vaccine utilizing a β toxoid preparation was introduced in the PNG highlands, where it reduced hospital admissions for PigBel by ~80% (Johnson and Gerding, 1997; Lawrence, 1997). This finding supports the importance of β toxin in the pathogenesis of this illness.

Epidemiology: *C. perfringens* Veterinary Enterotoxemias

Besides its broad distribution in the environment, particularly in soil, *C. perfringens* is very frequently found in the digestive tract of clinically healthy animals (Bullen, 1952; Niilo, 1980; Van Baelen and Devriese, 1987; Tschirdewahn et al., 1991; Ardehali et al., 1994). Most intestinal diseases produced by *C. perfringens* in animals are generically called "enterotoxemias," a term that implies production of toxins in the gastrointestinal tract and absorption into the general circulation with systemic effects. This is true for some of the intestinal infections by *C. perfringens* (for example *C. perfringens* type D infection in sheep) but not for other *C. perfringens* infections, in which most damage is caused by a toxin (or toxins) that is produced, and remains, in the intestines (for instance, *C. perfringens* type D enterotoxemia in adult goats). As a general rule, clostridial veterinary enterotoxemias have a low incidence but a high mortality.

Clostridium perfringens Type A *Clostridium perfringens* type A is the most ubiquitous of the *C. perfringens*; it is found in most soils and is also highly prevalent in the intestine of most warm-blooded animals (Itodo et al., 1986; Songer, 1996; Uzal and Marcellino, 2002a). In different surveys of the types of *C. perfringens* present in the intestine of healthy ruminants and soil, *C. perfringens* type A was found in 51–86.6% of the samples examined (Itodo et al., 1986; Uzal and Marcellino, 2002a). Because *C. perfringens* type A is highly prevalent in the intestines of healthy animals, controversy exists about its real pathogenic role.

An exception to this uncertainty, however, is with chickens, where there is considerable evidence that *C. perfringens* type A is responsible for necrotic enteritis. In this species, damage to the intestinal mucosa by high fiber diets or concurrent infection by coccidia are the most likely predisposing factors for necrotic enteritis (Nairn and Bamford, 1967; Helmboldt and Bryant, 1971; Al-Sheikhly and Al-Saieg, 1980; Shane et al., 1985; El-Seedy, 1990; Songer, 1996). It has also been demonstrated that germ-free chickens have higher mortality than conventional chickens (Fukata et al., 1988; Fukata et al., 1991), which suggests that the normal intestinal flora

plays a role in the pathogenesis of this disease. Cholangiohepatitis in chickens is also believed to be produced by *C. perfringens* type A. While it has been assumed that the risk factors for this disease are the same as those for necrotic enteritis, this has not been confirmed (Onderka et al., 1990).

Clostridium Perfringens Types B and C Type B produces diseases in several animal species and can be found, although infrequently, in the intestine of healthy animals (Onderka et al., 1990; Uzal and Marcellino, 2002a). This type of *C. perfringens* has been found in 4.25–7.1% of healthy ruminants in different surveys (Itodo et al., 1986; Uzal and Marcellino, 2002a). For reasons that are not very clear, disease caused by *C. perfringens* type B has been reported only in a few regions of the world. This type of *C. perfringens* affects mainly sheep, and disease in this species has been reported in the United Kingdom, South Africa, the Middle East (Timoney et al., 1988) and the United States (Tunnicliff, 1933). However, *C. perfringens* type B has also been isolated from intestinal contents of healthy animals in countries or regions where disease has not been reported (Uzal and Marcellino, 2002a). Although it is possible that the lack of reported diagnosis in some regions or countries of the world is due to diagnostic difficulties, it would also appear that other factors, in addition to the presence of the microorganism, are necessary for disease to occur.

Type C has been isolated from a great variety of species associated or not with clinical disease (Timoney et al., 1988; Songer, 1996). In surveys of healthy ruminants, this microorganism was isolated from the intestinal content of 4.9% of the samples in one study (Itodo et al., 1986), while no *C. perfringens* type C was isolated in another (Uzal and Marcellino, 2002a). Infections by *C. perfringens* type C have been reported in sheep, cattle, pigs, horses, chickens, dogs and humans in several countries (Mackinnon, 1989; Songer, 1996).

The main pathogenic factor of *C. perfringens* types B and C is β toxin (Table 2). Beta toxin is very sensitive to the action of trypsin, and newborn animals are usually the most susceptible to infection by these microorganisms, since little protease activity is present in the gut of these animals (Barker et al., 1993). Also, it has been suggested that newborn animals are more often infected by *C. perfringens* types B and C because of the ready colonization of the gut by *C. perfringens* in the absence of well established normal intestinal flora (Songer, 1996). As with the other *C. perfringens* types, dietary changes may also be a predisposing factor in infections by *C. perfringens* types B and C (Timoney et al., 1988).

CLOSTRIDIUM PERFRINGENS TYPE D Disease from *C. perfringens* type D has been reported in several species, but it is most prevalent in sheep and goats. Despite the common belief that this microorganism is a normal inhabitant of the intestine of most ruminants, different surveys isolated *C. perfringens* type D from less than 20% of the animals, and there were farms where it was not isolated at all (Itodo et al., 1986; Uzal and Marcellino, 2002a).

In sheep, enterotoxemia by *C. perfringens* type D has been reported in most sheep producing areas of the world (Carrillo et al., 1982; Lewis, 2000). The disease seems to be more prevalent in animals 3 weeks to 10 months old (Blood et al., 1983), but it can occur throughout the entire life of the animals. In this species, most enterotoxemia cases occur soon (a few hours to a few days) after sudden changes in diet, usually to diets rich in highly fermentable carbohydrates (Bullen, 1952; Lewis, 1998; Lewis, 2000), which promote the overgrowth of *C. perfringens* type D present in the intestine of healthy animals. Examples of this are the introduction of animals to feedlots without progressive adaptation to grains or concentrated rations, and access to lush grass after rains in spring and autumn. Overeating also appears to play an important role in the pathogenesis of enterotoxemia (Bullen, 1963). Because of these predisposing factors, fat, well-fed animals are particularly susceptible (Blood et al., 1983). Other factors that disturb the intestinal environment have also been suggested as predisposing factors for enterotoxemia in sheep, although no final proof of this has been reported. Among these is heavy tape-worm infestation (Thomas et al., 1956; Elliot, 1986) and dosing with phenothiazine (Jansen, 1960).

Morbidity rates can vary but seldom exceed 10% (Blood et al., 1983). The disease is almost 100% lethal (Hartley, 1956; Blood et al., 1983; Lewis, 2000).

Caprine enterotoxemia by *C. perfringens* type D occurs worldwide in goats of any age older than two weeks (Baxendell, 1988). The factors predisposing goats to enterotoxemia by *C. perfringens* type D have not been well defined, although it is usually assumed that, as in the case of sheep, any factor that increases the amount of starch in the small intestine could predispose goats to the disease. Factors such as sudden changes from poor to lush pasture, feeding of bread or other bakery goods, feeding of a bran/molasses mash, excessive concentrates consumption and feeding of garden greens to goats unaccustomed to green feed have been reported (King, 1980).

In addition to the dietary factors, other prerequisites seem to be necessary for the development of enterotoxemia in goats. For instance, Smith and Sherman (1994) reported an outbreak of enterotoxemia in a herd of goats that had been fed a consistent diet of hay and concentrates for many months before the outbreak. In cases like this, it is possible that errors in the mixing of the rations may have been responsible for an overdose of grain in some goats. Also, some outbreaks of enterotoxemia type D have been reported to occur in goats under extensive grazing systems without known diet change (Van Tonder, 1975; Uzal et al., 1994). An accidental overdose of an antihelmintic, cold weather stress, and a concomitant infestation with coccidia were suggested as possible predisposing factors in one outbreak of caprine enterotoxemia (Uzal et al., 1994). Heavy worm burden has also been reported as a predisposing factor for enterotoxemia in goats (King, 1980).

Individual sporadic cases or outbreaks of enterotoxemia with high morbidity have been observed (Smith and Sherman, 1994; Uzal et al., 1994). The persistence of *C. perfringens* type D in the environment, derived from previous cases of enterotoxemia, or from constant fecal contamination from animals in which this microorganism is part of the normal intestinal flora, could be an explanation for this disease pattern, although sudden changes in diet, as in the case of sheep, are most likely to be the major predisposing factors (Smith and Sherman, 1994). Although *C. perfringens* type D is usually considered to be a normal inhabitant of the alimentary tract of goats (Bullen, 1952; Niilo, 1980), there have only been a few documented reports of the isolation of *C. perfringens* type D from the intestinal tract or feces of healthy goats (Sinha and Kuppuswamy, 1969; Sinha, 1970).

Despite a generalized belief among producers and veterinarians that many unexplained sudden deaths in cattle are due to type D enterotoxemia (Blood et al., 1983), laboratory evidence does not seem to support such field observations, and there are only a few reports (Munday et al., 1976) about this condition in cattle. Epidemiological information about type D enterotoxemia is therefore almost nonexistent, although it is commonly assumed that the same factors that predispose sheep and goats to *C. perfringens* type D enterotoxemia, are predisposing factors for the disease in cattle (Barker et al., 1993).

CLOSTRIDIUM PERFRINGENS TYPE E Information about the prevalence of *C. perfringens* type E in the environment and in the intestine of healthy animals is scant. In a survey of this microorganism in the intestine of sheep, cattle and soil in Nigeria, *C. perfringens* type E was isolated from 7.9% of the samples (Itodo et al., 1986).

With the exception of the rabbit, epidemiological information about *C. perfringens* type E infection in animals is almost nonexistent. Although for many years, *C. perfringens* type E was regarded as the etiological agent of rabbit enterotoxemia, it is possible that many of those cases may have been due to *C. spiriforme*, since antitoxin prepared against *C. perfringens* type E τ toxin will also neutralize similar toxins produced by *C. spiriforme* (Songer, 1996; Percy and Barthold, 2001). The pathogenesis of this disease is believed to be similar to the carbohydrate overload model explained for sheep and goats; rabbits ingesting high energy feed may fail to degrade and digest the majority of carbohydrates in the small intestine. The majority of carbohydrates may then reach the large intestine promoting the overgrowth of *C. perfringens* (Percy and Barthold, 2001).

Clinical Illness: *C. perfringens* Gastrointestinal Diseases of Humans

CLOSTRIDIUM PERFRINGENS TYPE A FOOD POISONING

Disease As illustrated in Fig. 13, this food poisoning typically develops after ingestion of foods that are grossly contaminated (~10^6 bacteria/gram of food) with vegetative cells of a *C. perfringens* type A isolate carrying a chromosomal *cpe* gene (McClane, 2001a). Many ingested vegetative cells are killed by the acidity of the stomach, but when sufficient numbers of vegetative cells are ingested, some survive and pass into the small intestines. After initially multiplying in the intestines, these bacteria soon commit to sporulation. The trigger for this in vivo sporulation may involve (McClane, 2001a) an acid-shock response (in the stomach) and/or a response to bile exposure (in the intestines).

It is during sporulation in the intestines that *C. perfringens* expresses the enterotoxin, which then accumulates in the cytoplasm of the mother cell (McClane, 2001a). Therefore, CPE is not a secreted protein but instead is released into the intestinal lumen at the conclusion of sporulation as the mother cell lyses to release its mature spore. The enterotoxin then binds and exerts its molecular action (described in the Toxins section of this chapter) to kill enterocytes via apoptosis and/or oncosis, depending on CPE dosage. This cytotoxic effect produces histopathologic damage that begins at the villus tip and progresses to include severe epithelial desquamation and villus shortening. CPE-induced intestinal histopathologic damage can appear within 30 min of toxin treatment and appears responsible for initiating intestinal fluid and electrolyte losses (Sherman et al., 1994). With time, CPE-induced paracellular permeability effects and/or inflammation (see the Toxins section) may also contribute to intestinal electrolyte and fluid losses.

In rabbit models, CPE is active on all segments of the small intestine, with the ileum being particularly sensitive (McDonel, 1986). In this animal model, CPE does not affect the large intestine; however, it remains unclear whether the human colon is CPE-sensitive.

Common symptoms of *C. perfringens* type A food poisoning include diarrhea and abdominal cramps (McClane, 2001a). These symptoms usually begin about 12 h after ingestion and then persist for 12–24 h. In most cases a victim makes a full recovery, although this food poisoning can be fatal (see the Epidemiology section). Expo-

Clostridium perfringens Food Poisoning

Fig. 13. The pathogenesis of *C. perfringens* type A food poisoning. A food item (usually a meat or poultry item) becomes contaminated with vegetative cells of a type A isolate carrying a chromosomal *Clostridium perfringens* enterotoxin (*cpe*) gene. After ingestion, those bacteria sporulate in the intestines. During that in vivo sporulation, CPE is produced. The toxin is then released into the lumen at the completion of sporulation (when the mother cell lyses). Once free, the enterotoxin binds and, via membrane permeability alterations, kills enterocytes, producing intestinal damage that initiates diarrhea and cramping. From McClane (1992) with permission.
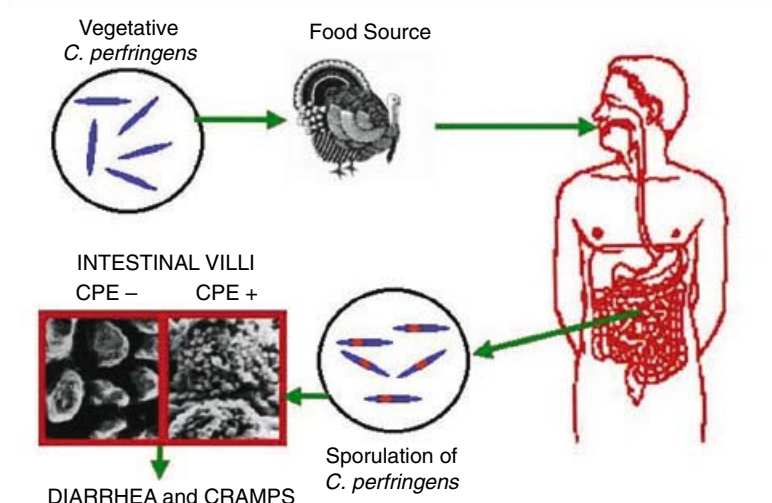
sure to *C. perfringens* type A food poisoning induces an immune response against the enterotoxin, but there is no evidence that previous exposure provides immunity.

Diagnosis Public health officials first use epidemiologic criteria (incubation time, symptoms, etc.) to evaluate the possible involvement of *C. perfringens* in a food poisoning outbreak (McClane, 2001a). However, given that the epidemiologic characteristics of *C. perfringens* type A food poisoning and the diarrheal form of *Bacillus cereus* food poisoning are indistinguishable, laboratory analyses often prove pivotal for diagnosis. Quantitative bacteriologic analysis of feces were traditionally used to identify a *C. perfringens* type A food poisoning outbreak, but that approach is imprecise, i.e., many healthy people, particularly the elderly, carry large numbers of (*cpe*-negative) *C. perfringens* isolates in their feces (McClane, 2001a). Both the CDC and United States Food and Drug Administration (FDA) now also use fecal CPE detection from two or more food poisoning victims as a diagnostic criterion for identifying a *C. perfringen*s type A food poisoning outbreak. Several commercial serologic CPE detection assays (enzyme-linked immunosorbent assays [ELISAs] and restriction fragment length polymorphism [RFLP] assays) are available for this purpose. It should be emphasized that CPE detection works best when fecal samples are collected soon after the onset of illness; with time, fecal CPE levels fall markedly and are more difficult to detect (McClane, 2001a).
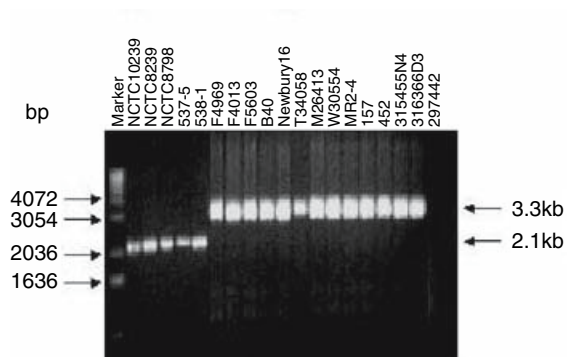
Fig. 14. Duplex polymerase chain reaction (PCR) *Clostridium perfringens* enterotoxin gene (*cpe*) genotyping assay. Results obtained using purified DNA extracted from type A isolates carrying a chromosomal *cpe* locus (lanes 2–6 from left) or plasmid *cpe* locus (next 13 lanes). Also shown are negative results using *cpe*-negative type A isolate 297442 (far right lane). Arrows at right depict migration of ~3.3-kb PCR product from chromosomal *cpe* locus or ~2.1-kb PCR product from plasmid *cpe* locus of type A isolates. Numbers at left indicate the migration of molecular size markers. From Wen et al. (2003) with permission.

For epidemiologic investigations, it can also be useful to demonstrate the presence of *cpe*-positive *C. perfringens* isolates in foods or feces associated with a food poisoning outbreak. For this purpose, a multiplex polymerase chain reaction (PCR) assay is available (Garmory et al., 2000). A *cpe* genotyping PCR can then be used to specifically determine whether those food or fecal isolates carry the chromosomal *cpe* gene that is strongly associated with food poisoning (Fig. 14).

Treatment No specific treatment is used for cases of *C. perfringens* type A food poisoning. As for all diarrheal diseases, supportive fluid and electrolyte restoration is important.

CPE-Associated Nonfoodborne Human GI Diseases

Disease Clostridium perfringens type A strains carrying a *cpe* plasmid cause several nonfoodborne human GI diseases (Carman, 1997a), including antibiotic-associated diarrhea (AAD) and sporadic diarrhea (SD). Some evidence suggests CPE-associated AAD/SD, which often occur in institutions such as hospitals or nursing homes, result from person-to-person transmission or ingestion of environmental contaminants (Borriello et al., 1985c). However, the possibility that these diseases also result from antibiotic-induced overgrowth of normal flora cannot yet be ruled out.

Estimates vary considerably between surveys, but CPE-associated AAD and SD apparently causes from 1% up to 5–20% of all cases of these human GI illnesses (Carman, 1997a). The pathogenesis of CPE-associated AAD and SD remains poorly understood, but their symptoms include diarrhea and cramping (Borriello et al., 1984; Borriello, 1995). These symptoms are often more severe and longer lasting (up to several weeks) than the typical GI symptoms of *C. perfringens* type A food poisoning. These differences could result from transfer of the *cpe* plasmid from the few infecting *cpe*-positive strains initiating AAD and SD to the many *cpe*-negative *C. perfringens* isolates present in the normal intestinal flora (Brynestad et al., 2001). As those normal flora isolates are presumably under selection for colonization, this putative in vivo virulence conversion via *cpe* plasmid transfer could produce fully pathogenic isolates with enhanced colonization ability.

CPE-associated AAD and SD are most common in the elderly (Borriello et al., 1984; Carman, 1997a). The extent of fatalities from these infections is not clear at present.

Diagnosis Most cases of CPE-associated AAD/SD are initially identified by fecal CPE detec-

tion. Therefore, it becomes important to distinguish whether a case involves food poisoning or AAD and SD, e.g., can a contaminated food source be identified? Genotyping of isolates (Fig. 14) to determine if they carry the plasmid *cpe* gene typically associated with AAD and SD is also helpful, but not foolproof, as an atypical food poisoning outbreak involving plasmid *cpe* isolates was recently identified in Japan (Tanaka et al., 2003).

Treatment In most cases of CPE-associated AAD/SD, only symptomatic therapy to restore fluid/electrolyte balance is necessary. For severe cases, metronidazole has been successfully used (Borriello et al., 1984; Borriello, 1985a; Borriello et al., 1985c; Borriello, 1995).

Enteritis Necroticans

Disease The pathogenesis of PigBel is now fairly well understood (Lawrence, 1997). People in the PNG highlands eat a diet rich in sweet potatoes, which contain a potent trypsin inhibitor. This low protein diet also reduces their production of pancreatic proteases, including trypsin. These predisposing factors become important on special occasions when in PNG typically a pig is killed and cooked (often under unsanitary conditions) for consumption by the celebrants. A pork vehicle is an important contributing factor for the development of PigBel as *C. perfringens* type C isolates are often present in pigs. In incompletely cooked foods, these bacteria survive and, once ingested, multiply and produce β toxin in vivo. Normally this toxin would simply be inactivated by intestinal trypsin, but in the PNG highlands population, this does not occur owing to the presence of trypsin inhibitor (from sweet potatoes) and low trypsin levels (from a protein-poor diet) present in that population. Consequently, β toxin remains active in the intestines, where it induces intestinal necrosis. The question remains whether β toxin is the only toxin involved in the pathogenesis of enteritis necroticans caused by type C isolates, since purified β toxin alone does not induce substantial intestinal damage.

In developed countries, most cases of enteritis necroticans occur in people with pancreatic diseases affecting production of trypsin (Johnson and Gerding, 1997). Poor diet and/or low intestinal motility can also be contributing factors.

The primary symptom of enteritis necroticans is abdominal pain (Johnson and Gerding, 1997). In PNG, this pain typically develops ~1–5 days after eating the protein-rich meal (i.e., pork); the most severe cases develop very quickly after eating the contaminated food product. There may also be vomiting and bloody diarrhea. In severe cases of the disease, intestinal obstruction due to necrosis of the small bowel (particularly the jejunum) develops. Affected intestinal tissue shows necrosis and hemorrhaging, with polymorphonuclear leukocyte (PMN) infiltration.

Death can occur from toxemia (Johnson and Gerding, 1997). Some cases are so rapidly fatal that the individual never makes it to the hospital. Prior to the introduction of a β toxoid vaccine in 1980, PigBel ranked as the second leading cause of death in PNG children after weaning.

Diagnosis Enteritis necroticans is diagnosed solely on the basis of clinical signs (Johnson and Gerding, 1997). The abdomen is typically distended and thickened loops of bowel are often palpable on examination.

Treatment Management of enteritis necroticans involves a combination of nasogastric suction, intravenous fluids, antimicrobial therapy and, frequently, surgical resection of the affected bowel segment (Johnson and Gerding, 1997). A number of antimicrobials have been used, including penicillin, chloramphenicol and metronidazole. The most important factor for successful management of this disease is the timing of surgery. Patients are usually observed for 1–2 days; if there is no improvement, laparotomy is performed (this surgical procedure can be performed even earlier for severe cases). Delaying surgery can have a negative outcome.

Clinical Illness: *C. perfringens* Veterinary Enterotoxemias

Disease Veterinary enterotoxemias caused by *C. perfringens* are the consequence of the local or systemic action of the toxins produced by this microorganism in the gastrointestinal tract. The main features of the most important enterotoxemias produced by each *C. perfringens* type in the most relevant domestic animal species are discussed here.

Clostridium perfringens type A

Sheep *Clostridium perfringens* type A produces a rare form of enterotoxemia in lambs that has been described in California and Oregon in the United States (McGowan et al., 1958). This condition, also known as "yellow lamb disease" (McGowan et al., 1958), is characterized clinically by depression, anemia, icterus, hemoglobinuria and acute death (McGowan et al., 1958; Songer, 1996). Postmortem changes consist of generalized icterus (Fig. 15) and enlarged, pale and friable livers. Red tinged urine is found in the urinary bladder (McGowan et al., 1958).

Fig. 15. Yellow lamb disease by *Clostridium perfringens* type A. Diffuse icterus.

Fig. 16. Hemorrhagic bowel syndrome. Serosal view (A) and cross-section of small intestine (B). Photograph courtesy of Deryck Read.

Fig. 17. Hemorrhagic colitis in a horse (colitis X). The role of *C. perfringens* in this condition has not been proven. Photograph courtesy of Roger Kelly.

Goats A condition clinically similar to yellow lamb disease has been reported (Russell, 1970), but definitive confirmation of the role of this microorganism in disease production has not been reported (Barron, 1942; Russell, 1970; Guss, 1977; Songer, 1998).

Cattle Though producers, veterinarians and vaccine producers claim *C. perfringens* type A is important in diseases of cattle (Manteca et al., 2002; F. A. Uzal, unpublished observations), this microorganism's role in disease production in this animal has not been firmly established (Songer, 1996; Petit et al., 1999). *Clostridium perfringens* type A has been isolated in the western United States from calves with tympany, diarrhea and high mortality (Songer, 1996). At postmortem examination, hemorrhage and ulceration of the abomasal mucosa, necrohemorrhagic enteritis of the jejunum, ileum and sometimes colon are the most striking gross changes (Songer, 1996). Although *C. perfringens* isolates originating from cases of enteritis in cattle were highly enterotoxigenic type A strains (Niilo, 1978), Koch's postulates have not been fulfilled (Songer, 1996), and the role of *C. perfringens* type A in disease of cattle is not yet defined. It has also been suggested that *C. perfringens* type A is involved in the pathogenesis of a condition in cattle called "hemorrhagic bowel syndrome" (Denison et al., 2002; Fig. 16), but to date no evidence has been provided to confirm this hypothesis. Further research is necessary to clarify the roles of different predisposing factors, this microorganism, and its toxins in disease pathogenesis in cattle.

Horses *Clostridium perfringens* type A has been suggested to be the etiological agent of a condition of horses called "colitis X," characterized by watery or bloody diarrhea and hemorrhagic colitis (Barker et al., 1993; Fig. 17). Large numbers of *C. perfringens* type A have been found in the gut of these animals, although a definitive role of this microorganism in colitis X has not been proved (Songer, 1996). The action of other bacteria (such as *Salmonella* sp.) as primary patho-

gens and proliferation of *C. perfringens* as a result of disruption of the bacterial ecosystem in the colon has been hypothesized for this condition (Barker et al., 1993).
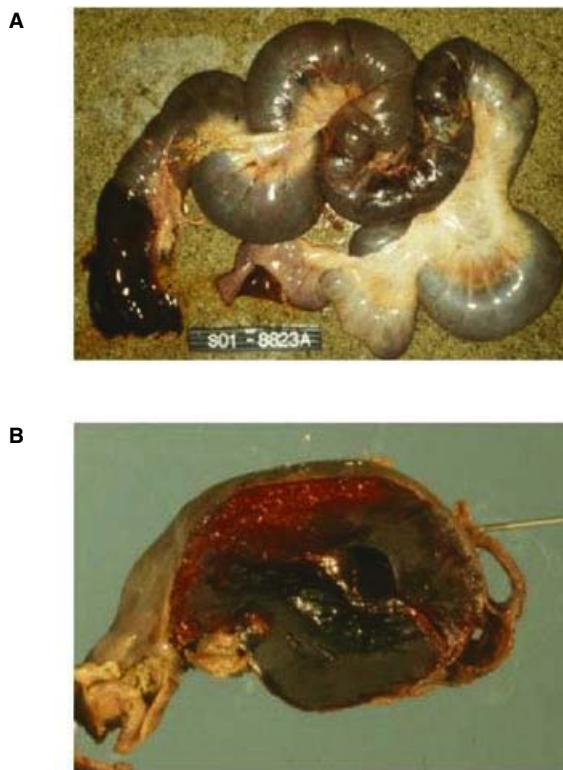
Chickens *Clostridium perfringens* type A produces necrotic enteritis in chickens (Long and Truscott, 1976; Niilo, 1978; Baba et al., 1992). Under appropriate conditions (high fiber diets, concurrent coccidia infection, and reduced intestinal flora), the organism replicates readily in the intestinal tract, where it produces the α toxin believed to be responsible for the clinical signs and pathological changes of the disease (Ficken et al., 1997). The disease usually takes an acute course, although subclinical disease that does not result in mortality but is associated with poor performance can also be observed (Prescott, 1979; Kaldhusdal and Hofshagen, 1992). Postmortem lesions consist of dilated small intestine (jejunum and ileum) with dark brown granular content (Fig. 18), mucosal necrosis (Nabuurs et al., 1983; Ratz et al., 1989), and catarrhal colitis (Ratz et al., 1989) with a large number of Gram-positive rods observed. Alpha toxin's role in this disease is supported by observations that duodenal infusion or oral inoculation of chickens with preparations of this toxin can reproduce the lesions of intestinal necrosis (Al-Sheikhly and Truscott, 1977; Fukata et al., 1991).

Clostridium perfringens type A has also reportedly produced cholangiohepatitis in chickens (Onderka et al., 1990), with or without necrotic enteritis (F. A. Uzal, unpublished observations). Cholangiohepatitis alone can cause downgrading of chickens at slaughter. However, when cholangiohepatitis occurs alone, it presents as a subclinical condition, and no disease has been reported in on-farm stocks (Onderka et al., 1990). Gross pathological changes consist of pale spots on the superficial (Fig. 19) and cut surfaces of the liver, which histologically correspond to multifocal hepatitis with bile duct proliferations, multiple granulomas, and degeneration of hepatocytes (Onderka et al., 1990). A condition with gross and histological lesions identical to the natural condition has been experimentally reproduced in chickens by either tying off the bile ducts or injecting *C. perfringens* into the ducts (Onderka et al., 1990).

South American Camelids It has been suggested that *C. perfringens* enterotoxemia is the most serious disease of neonate alpacas in Peru (Fowler, 1998). Animals are generally 3–80 days of age when affected, the onset of disease is rapid, and lethality is almost 100% (Fowler, 1998). Adults are rarely, if ever, affected. Clinically, the disease is characterized by sudden death or neurological signs, while the most strik-

Fig. 18. Necrotic enteritis of chickens by *C. perfringens* type A.

Fig. 19. Cholangiohepatitis of chickens by *C. perfringens* type A.

ing gross postmortem changes are pulmonary edema, serosal petechyation, edema and hyperemia of the intestinal tract mucosas with the intestine distended with watery fluid (Fowler, 1998).

Other Animal Species Disease by *C. perfringens* type A has also been reported in minks (Macarie et al., 1980), dogs (Songer, 1996), muskrats (Vustina, 1988), camels (Seifert et al., 1992), ferrets (Songer, 1996) and water buffaloes (Worrall et al., 1987).

Clostridium perfringens Types B and C

Sheep *Clostridium perfringens* types B and C produce similar diseases in sheep from a clinical point of view. These illnesses are characterized by sudden death or acute neurological signs with or without hemorrhagic diarrhea (Barker et al., 1993; Lewis, 2000). Lamb dysentery and hemorrhagic enteritis (*C. perfringens* type B)

occur in lambs under 3 weeks of age, while struck (*C. perfringens* type C) is a condition of adult sheep (Lewis, 2000).

Lamb dysentery usually affects strong lambs that ingest large quantities of milk. Offspring of heavy milking breeds are therefore most vulnerable (Lewis et al., 1995; Lewis, 1998). The clinical course is seldom longer than a few hours, during which the lamb has abdominal pain, stops suckling, collapses and dies, its feces being semi-fluid and blood-stained. Occasionally the animal survives a few days and can display central nervous signs (Mitchell and Linklater, 1983; Songer, 1996; Lewis, 2000).

Struck is a condition of adult sheep caused by *C. perfringens* type C (Barker et al., 1993; Lewis, 2000). Clinically the disease is characterized by sudden death after a short illness in which diarrhea and signs of abdominal pain can be observed. A disease clinically and pathologically very similar to the natural condition has been successfully reproduced in maturing lambs by intraduodenal infusion of *C. perfringens* type C supplemented with soybean flour as a protease inhibitor (Niilo, 1986).

Clostridium perfringens types B and C in sheep produce similar intestinal lesions consisting of diffuse or multifocal hemorrhagic enteritis, predominantly in the ileum (Fig. 20), with excess sanguineous serous fluid in the abdominal cavity and serosal petechias (Barker et al., 1993). In older animals with lamb dysentery or hemorrhagic enteritis that have survived for a few days, focal symmetrical encephalomalacia (FSE) may be present owing to the action of toxin (Barker et al., 1993; Lewis, 2000).

Goats In goats, descriptions of enterotoxemia by *C. perfringens* types B and C are scant, not very well documented, and definitive confirmation of their role in disease production has not been published (Barron, 1942; Guss, 1977; Songer, 1998).

Cattle Enteritis by *C. perfringens* type C is a rare diagnosis in feedlot cattle less than 10 days of age, characterized clinically by abdominal pain and sometimes neurological signs with acute death (Griner and Bracken, 1953). Lethality is usually close to 100%. The gross and microscopic changes of this condition are similar to those of struck in sheep (Barker et al., 1993).

Horses *Clostridium perfringens* type C causes necrotic enteritis in foals (Dickie et al., 1978; Howard-Martin et al., 1986; Pearson et al., 1986; Drolet et al., 1990; Songer, 1996). Clinically the disease is characterized by an acute course, although it can last as long as two days. Affected animals display depression and have hemorrhagic diarrhea accompanied with dehydration (Howard-Martin et al., 1986). Colic can be occasionally observed (Howard-Martin et al., 1986). At postmortem examination, acute fibrinonecrotic enteritis, multifocal or diffuse, is observed in the jejunum and ileum (Barker et al., 1993; Fig. 21). Large numbers of Gram-positive rods can be seen in mucosal smears and histological sections (Barker et al., 1993; Songer, 1996; Songer, 1996); however, when the animals have survived for relatively long periods, other bacte-

Fig. 20. Lamb dysentery by *C. perfringens* type B. Serosal (A) and mucosal view (B).

Fig. 21. Necrotic enteritis by *C. perfringens* type C in a foal.

ria may colonize the intestinal lesions making the interpretation of Gram-stained smears and histological sections confusing (Manteca et al., 2002; F. A. Uzal, unpublished observations). Terminal toxemia may result in widespread edema and hemorrhages (Barker et al., 1993).

Pigs *Clostridium perfringens* type C produces necrotic enteritis in neonatal pigs. It has been stated that the pig is the most commonly affected species by the type C of this microorganism (Fitzgerald et al., 1988; Johnson et al., 1992; Pace et al., 1992; Songer, 1996). The disease can have up to 50% morbidity in a given herd (Niilo, 1988; Ohnuna et al., 1992) and lethality of 50–100% (Songer, 1996). Clinically, the disease is acute or peracute, with a clinical course lasting less than 24 h, in 1–2 day-old piglets (Mackinnon, 1989). Depression and bloody diarrhea are the main clinical signs in young piglets, although older animals can have diarrhea without blood (Niilo, 1988). Postmortem findings consist of necrotic enteritis with thick hemorrhagic exudate in the lumen. The necrosis of the intestinal wall can affect all layers of the intestine (Field and Gibson, 1955; Arbuckle, 1972; Mackinnon, 1989; Cho et al., 1991; Ohnuna et al., 1992). High intestinal counts of *C. perfringens* are a common finding in this condition (Songer, 1996).

Other Species Gastrointestinal disease has been reported in dogs and chickens with *C. perfringens* type C infection (Songer, 1996) and in South American camelides (Fowler, 1998).

Clostridium perfringens Type D

Sheep *Clostridium perfringens* type D produces a peracute to chronic neurological condition in sheep. The condition is characterized clinically by sudden death or acute to chronic neurological signs including blindness, opisthotonos, convulsions, bleating and recumbency. Respiratory difficulty is an almost constant clinical sign. Diarrhea can occasionally be observed, although this is not a common clinical sign in sheep (Lewis, 2000; Uzal, 2004a, 2004b).

Several gross findings such as excess pericardial fluid with or without fibrin strands (Fig. 22), excess pleural fluid, serosal and pericardial petechyation (Fig. 23) and lung edema (Fig. 24) are common gross postmortem changes of the disease in sheep (Barker et al., 1993; Uzal et al., 2004b). Relatively mild intestinal changes consisting of hyperemia of the small intestine with slight to markedly red fluid contents (Fig. 25) and occasional colitis have been reported in some cases of type D enterotoxemia in sheep (Barker et al., 1993). However, contrary to the popular belief among many producers and veterinarians,
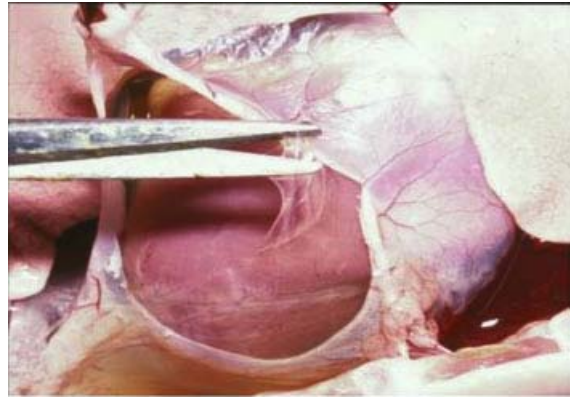
Fig. 22. Excess of pericardial fluid with fibrin strands in a lamb with type D enterotoxemia.
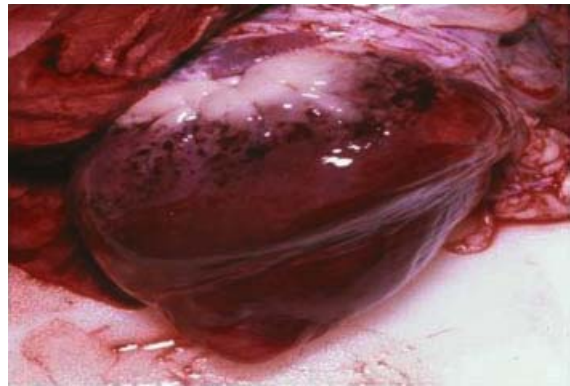
Fig. 23. Epicardial petechiae in a lamb with type D enterotoxemia.

gross intestinal changes are rarely present in sheep enterotoxemia by *C. perfringens* type D. The kidney lesion from which the disease gets its name (pulpy kidney disease) is considered to be a postmortem change (Barker et al., 1993; Uzal et al., 2004b) and is not seen in animals recently dying from type D enterotoxemia (Uzal et al., 2004b)

In a relatively low percentage of acute cases of the disease, herniation of the cerebellar vermis through the foramen magnum of the occipital bone can be seen, while in chronic cases of the disease FSE, a condition characterized by dark-hemorrhagic foci in corpus striatum, thalamus, midbrain and cerebellar white matter cores (Hartley, 1956; Buxton and Morgan, 1976; Buxton et al., 1978b) can be seen. Less consistently, FSE has also been found in cerebral cortex, pons, medulla and white matter of the occipital pole (Buxton et al., 1978b). Hyperglycemia and glycosuria can be found in approximately 50% of affected animals (Uzal et al., 2004b).
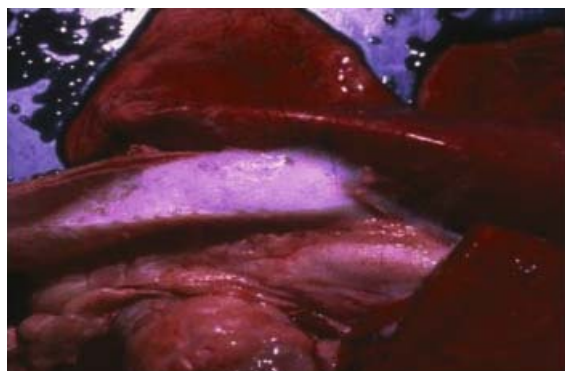
Fig. 24. Stable froth in the trachea indicating lung edema in a lamb with type D enterotoxemia.

Fig. 26. Proteinaceous perivascular edema in the basal ganglia of a sheep with *C. perfringens* type D enterotoxemia. Hemotoxylin and eosin, 125×.

Fig. 25. Mild diffuse hyperemia of the small intestine in a lamb with *C. perfringens* type D enterotoxemia.

Fig. 27. Focal symmetrical encephalomalacia in the cerebellar peduncles of a lamb with type D enterotoxemia. HE stain, 12×.

Histologically, the most consistent change of *C. perfringens* type D enterotoxemia in sheep is perivascular proteinaceous edema of the brain (i.e, acidophilic lakes of protein surrounding small and medium sized arteries and veins; Buxton et al., 1978b; Hornitzky and Glastonbury, 1993; Fig. 26). This change can be seen in animals a few hours after the onset of clinical signs and is also known as microangiopathy. The histological picture of FSE (Fig. 27) consists of degeneration of white matter, hemorrhage, astrocyte and axonal swelling and presence of vacuolated macrophages (gitter cells; Buxton et al., 1978b; Fig. 28). Although it is always assumed that microangiopathy is a constant change of type D enterotoxemia of sheep (Hornitzky and Glastonbury, 1993), most descriptions of microscopic lesions in the brain of sheep with enterotoxemia are based on experimental cases (Buxton and Morgan, 1976; Buxton et al., 1978b), and little information is available about these lesions in natural cases of the disease (Buxton, 1978a).

Buxton and Morgan (1976) suggested that brain lesions associated with toxin are due to the

Fig. 28. Large number of vacuolated macrophages (gitter cells) in an area of degeneration and malacia in the brain of a lamb with type D enterotoxemia. Hemotoxylin and eosin, 400×.

action of this toxin on endothelial cells, which generates perivascular edema followed by hypoxia and necrosis of the perivascular tissues. These authors suggested that toxin produced in

the small intestine and absorbed into the systemic circulation reacts with specific receptor sites in the brain of sheep, causing vascular endothelial cells to degenerate. This causes an alteration in fluid dynamics, which in turn causes the astrocyte end-feet to swell and rupture. Serum proteins and eventually red blood cells leak out and the resultant brain edema and hemorrhage produce the clinical nervous signs and the histopathological changes seen in cases of acute intoxication of sheep. However, Finnie et al. (1999) recently showed that toxin can also leak into the brain and act directly on rat neurons producing degeneration and necrosis of these cells in cases of subacute or chronic toxin intoxication. The intimate mechanism of action of toxin remains unknown and requires further investigation.

Goats Naturally occurring enterotoxemia by *C. perfringens* type D in the goat occurs in three forms: peracute, acute and chronic (Shanks, 1949; Oxer, 1956; Smith and Sherman, 1994). The peracute form occurs more frequently in young animals and is similar to the peracute form of the disease in sheep, with animals often found dead without clinical signs having been observed (Shanks, 1949; Oxer, 1956; Blackwell et al., 1991; Blackwell and Butler, 1992). The clinical course is usually less than 24 h and the lethality is always 100% (Smith and Sherman, 1994). The acute form is more frequently seen in adult goats (Smith and Sherman, 1994) and is characterized by diarrhea, abdominal discomfort, severe shock with cold extremities, opisthotonos and convulsions. The diarrhea may be initially yellow-green and pasty but rapidly becomes watery and mucoid with shreds of bowel mucosa and blood (Baxendell, 1988). This form of the disease usually results in death within 2–4 days after the onset of clinical signs, although in a few reported cases animals have recovered following treatment (Baxendell, 1988; Blackwell et al., 1991). The chronic form of the disease occurs in adult animals (Smith and Sherman, 1994), and clinical signs include profuse, watery diarrhea (often containing blood and mucus), weakness, anorexia, signs of abdominal discomfort, and agalactia (Blackwell and Butler, 1992). This form may last for days or weeks and is characterized by diarrhea and progressive weight loss (Shanks, 1949), and it may culminate in death or recovery (Blood et al., 1983).

As in sheep, hyperglycemia and glycosuria can be found in goats with enterotoxemia, although in a lower percentage than in sheep (approximately 25% of the animals) (Blackwell and Butler, 1992).

Gross postmortem changes can be completely absent in peracute cases of the disease, while an excess of pericardial fluid with or without fibrin, serosal hemorrhages, subendocardial hemorrhages, and a fibrino-necrotic colitis (Fig. 29) with colonic serosal edema are usually seen in acute cases of the disease. Diffuse, fibrino-necrotic colitis is the most prominent postmortem finding in chronic enterotoxemia of goats by *C. perfringens* type D (Blackwell et al., 1991; Barker et al., 1993; Uzal and Kelly, 1996).

Descriptions of histopathological changes in naturally occurring enterotoxemia of goats are scant. Microangiopathy and focal symmetrical degeneration have been observed in a few cases of peracute and acute enterotoxemia type D in goats, although these changes seem to be less common than in sheep (Blackwell et al., 1991; Uzal et al., 1997b). In acute and chronic cases of the disease, there is a fibrino-necrotic colitis (Fig. 30) with large numbers of Gram-positive rods (Blackwell et al., 1991; Blackwell and Butler, 1992). Although some authors have claimed that degeneration of the renal proximal tubular epithelium occurs in type D enterotoxemia of
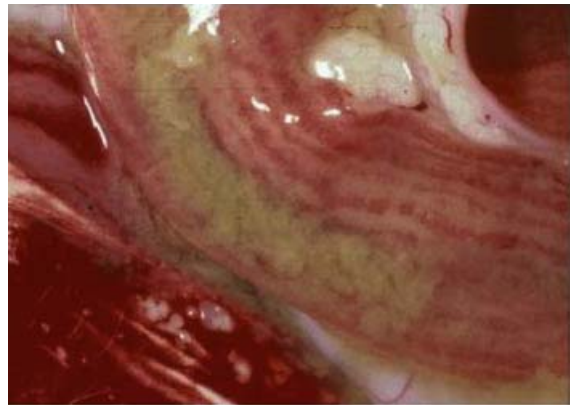
Fig. 29. Fibrino-necrotic colitis in a goat with *C. perfringens* type D enterotoxemia.
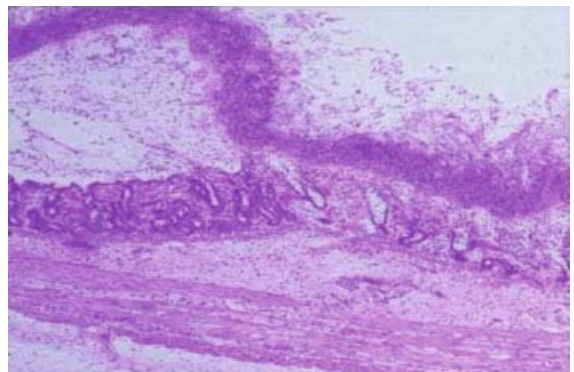
Fig. 30. Fibrino-necrotic colitis in a goat with chronic *C. perfringens* type D enterotoxemia. HE stain, 200×.

goats (Barker et al., 1993), this change has not been reported by most authors, and it is likely that, as in sheep, it represents a postmortem phenomenon.

It is not well understood why adult goats develop colitis, a lesion rarely seen in sheep type D enterotoxemia, and systemic lesions are rarely observed in these animals. Although it has been suggested that there could be a differential susceptibility in sheep and goats to the action of toxin on the small and large intestine (Blackwell et al., 1991), recent experiments have shown that when intestinal loops of sheep and goats are exposed to the action of toxin, they react in a similar way, namely colitis and no gross or histological changes in the small intestine (Fernandez Miyakawa and Uzal, 2002). The same experiment showed that fluid accumulation in the small intestine of animals treated with toxin is more marked in goats than in sheep. A more rapid toxin-induced accumulation of fluid in the small intestine of goats could more quickly flush away bacteria and toxin, moderating the intestinal toxin absorption. In contrast, the delayed physiological response in the small intestine of sheep might result in a greater absorption of toxin from the small intestine of this species. This hypothesis is reinforced by the fact that, when toxin is injected intravenously in kids or lambs, the clinical signs and the speed at which they develop are very similar in both species (Uzal and Kelly, 1997a). Other factors, such as the speed of toxin production by different strains of *C. perfringens* type D and the activity of other toxins, bacteria or parasites, might also be responsible for the different clinical signs observed in goats and sheep.

Cattle The literature on enterotoxemia in cattle is scant and sometimes contradictory. For instance, Barker et al. (1993) stated that lesions in calves dying of enterotoxemia caused by *C. perfringens* type D closely resembled those in lambs, including the brain changes typically described for sheep (Figs. 26–28). However, we could not find any firm support for this in the literature. The only report suggesting that brain lesions occur in bovine enterotoxemia is the description by Buxton et al. (1981) of brain lesion in calves, which closely resembled the FSE produced by *C. perfringens* type D toxin in sheep (Hartley, 1956). However, Buxton et al. (1981) did not attempt to test gut samples from their cases for the presence of toxin. Uzal et al. (2002b) injected calves intravenously with *C. perfringens* type D toxin and produced an acute neurological disease and histopathological lesions in the brain identical to those observed in acute enterotoxemia in sheep (microangiopathy; Fig. 31). These results indicate that cattle are
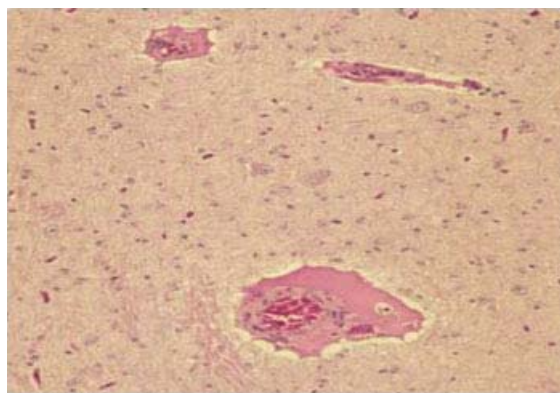
Fig. 31. Perivascular proteinaceous edema (microangiopathy) in the brain of a calf inoculated intravenously with *C. perfringens* type D toxin. HE stain, 125×.

susceptible to the action of toxin, but they do not necessarily imply that the disease occurs naturally in cattle.

Clostridium perfringens Type E

Sheep and Cattle *Clostridium perfringens* type E has been associated with intestinal disease in calves (Hart and Hooper, 1967; Meer and Songer, 1997; Billington et al., 1998), but it has been suggested that type E isolates are not a common cause of enterotoxemia in lambs (Songer, 1998). However, little information is available about this condition in these species.

Rabbits *Clostridium perfringens* type E is responsible for enterotoxemia in rabbits (Baskerville et al., 1980, Percy and Barthold, 2001). The disease has acute, subacute and chronic forms. Diarrhea and sudden death are the main clinical signs of the acute form. At postmortem examination, a carcass in good nutritional condition and perineal soiling with feces are usually the only gross findings in acute cases. In subacute and chronic cases, the carcasses may be thin and dehydrated and straw colored fluid may be present in the peritoneal cavity, together with ecchymosis on the cecal serosa, epicardium and thymus. The cecum is dilated with watery to mucoid content. The wall may be thickened due to submucosal edema. In acute cases, there are usually no histological changes and in the subacute and chronic forms, the most striking histological lesion is necrotizing typhlitis with relative sparing of the crypt bases and lamina propria and large number of Gram-positive rods (Percy and Barthold, 2001).

Clostridium perfringens Enterotoxin Some strains of all *C. perfringens* types, except type E, can produce enterotoxin (CPE; Songer, 1996;

Kokai-Kun and McClane, 1997b). Information about the role of CPE in human disease is plentiful, and CPE positive strains have been isolated from the intestine and feces of many animal species (Van Baelen and Devriese, 1987; Van-Damme-Jongsten et al., 1990). However, with the exception of the dog, very little scientific information is available about the role and pathogenesis of this toxin in diseases of domestic animals. Furthermore, intestinal contents or feces from sick or dead animals are very rarely investigated for the presence of CPE, and no diagnostic criteria have been established for CPE-produced diseases in most animal species.

Dogs This animal has been most studied in terms of the veterinary pathogenesis of CPE. In this species, hospital-acquired diarrhea is associated with CPE-positive *C. perfringens* (Carman and Lewis, 1983; Kruth et al., 1989).

Pigs CPE is not an infrequent finding in stools and intestinal content of pigs with diarrhea (Nabuurs et al., 1983; Popoff and Jestin, 1985; Collins et al., 1989; Estrada Correa and Taylor, 1989) and mild necrotic or degenerative changes in their small intestine (Nabuurs et al., 1983).

Foals The results of several studies suggest that CPE is responsible for intestinal disease (Kanoe et al., 1990; Staempfli et al., 1991). It has also been suggested that this toxin is involved in colitis X (Blood et al., 1983), although no evidence has been provided to either support or rule out this possibility.

Clostridium perfringens β2 Toxin The newly discovered *C. perfringens* β2 toxin has been associated with several diseases of animals (Gibert et al., 1997; Herholz et al., 1999; Gkiourtzidis et al., 2001; Manteca et al., 2002). However, to date, information about the role of this toxin in diseases of animals is scarce. *Clostridium perfringens* type A, producing α and β2 toxin, has been isolated from piglets and horses with necrotic enterocolitis (Gibert et al., 1997; Herholz et al., 1999). Because the role of α toxin in production of digestive lesions is controversial, it has been suggested that it is the β2 toxin that plays a role in causing digestive disease in horses (Herholz et al., 1999). A synergistic role of the α and β2 toxins in the production of necrotic and hemorrhagic enteritis in cattle has been postulated (Manteca et al., 2002). Beta2-positive *C. perfringens* strains were also isolated from lambs with dysentery, and it has been suggested that this toxin may also play a role on the production of this disease (Gkiourtzidis et al., 2001).

The implication of β2 toxin in animal disease is mostly based on a higher prevalence of β2-positive *C. perfringens* strains isolated from animals with enteritis than from normal controls (Gibert et al., 1997; Herholz et al., 1999; Manteca et al., 2002). Although these results suggest that β2 toxin could play an important role in the pathogenesis of enteric disease of several animal species, more research is necessary to clarify this. In particular, the use of this toxin in experimental animal models and the development of diagnostic techniques to detect the preformed toxin in clinical samples are necessary before the natural disease can be characterized and diagnostic criteria for infection involving β2-positive *C. perfringens* can be established.

DIAGNOSIS As mentioned before, *C. perfringens* can be a normal inhabitant of the intestines of most animal species (Niilo, 1965; Niilo, 1980; Itodo et al., 1986; Uzal and Marcellino, 2002a), including humans (Jansen, 1960). This fact makes the diagnosis of infections by *C. perfringens* challenging, and in most cases, the mere isolation of this microorganism from clinical specimens has no diagnostic value. Other criteria need to be taken into consideration for the diagnosis of infections by *C. perfringens* in animals. We discuss here the most commonly used criteria for the diagnosis of these infections.

Clinical History Since the pathogenesis of most *C. perfringens* infections involving rapid multiplication of the organism in the intestine with subsequent production of toxins, a history of sudden change in diet, particularly to a diet rich in carbohydrates, is usually suggestive of a possibility of enterotoxemia in most animal species (Bullen, 1952; Blood et al., 1983).

Prolonged antibiotic treatment is also an important precedent for consideration in cases of necrotic enteritis of horses, pigs and chickens (Songer, 1996).

Age is another important factor to consider when establishing a presumptive diagnosis of *C. perfringens* infection, as several of these conditions are age-specific. For instance, lamb dysentery from *C. perfringens* type B is almost exclusively observed in lambs that are one to a few days old, but *C. perfringens* type D enterotoxemia in this species is very rarely seen in lambs less than 2 or 3 weeks old (Lewis, 2000).

Vaccination history is frequently used by animal owners and veterinarians to rule out infections by *C. perfringens* (Uzal, 2004a). However, several factors (including variation in the quality of *C. perfringens* vaccines between countries and manufacturers and errors of transportation, storage and administration of these vaccines) make vaccination history not always reliable. In addi-

tion, inter- and intraspecies variation in intensity and duration of antibody responses occur frequently in several animal species (Bullen and Batty, 1957; Blackwell et al. 1983; Uzal et al., 1998b), while the kinetics of antibody production in many species is not known.

While no diagnosis of *C. perfringens* infections can be based on clinical history alone, the absence of these precedents should not, under any circumstance, be used to preclude a possible diagnosis of these diseases.

Clinical Signs Clinical signs are, at most, suggestive of *C. perfringens* infections (Uzal, 2004a), and no final diagnosis can be based on clinical grounds only. They vary according to the type of *C. perfringens* involved.

Clostridium perfringens Type A In sheep, sudden death, together with jaundice, can be suggestive of *C. perfringens* type A enterotoxemia (McGowan et al., 1958). However, there are several other more prevalent causes of jaundice in sheep.

Increased mortality with bloody diarrhea should be considered highly suggestive of necrotic enteritis by *C. perfringens* type A in chickens (Prescott, 1979; Kaldhusdal and Hofshagen, 1992). However, some cases of *C. perfringens* type A necrotic enteritis in chickens may not have bloody diarrhea (Songer, 1996).

In other animal species, until more information is available about the syndromes produced by *C. perfringens* type A, clinical signs are of little value in establishing a presumptive diagnosis of these infections.

Clostridium perfringens Types B and C In sheep, sudden death or acute neurological signs, with or without hemorrhagic diarrhea, are suggestive of *C. perfringens* type B or C infection (King, 1980; Lewis, 2000; Uzal, 2004a). These signs in lambs under 3 weeks of age are suggestive of lamb dysentery or hemorrhagic enteritis (*C. perfringens* type B), while similar signs in adult sheep are suggestive of struck (*C. perfringens* type C; King, 1980; Lewis, 2000; Uzal, 2004a). In pigs, high morbidity and mortality with bloody diarrhea in young pigs or bloodless diarrhea in older pigs are suggestive of *C. perfringens* type C infection (Songer, 1996). In other animal species, clinical signs of *C. perfringens* type C infection are of little value in establishing a presumptive diagnosis of this disease.

Clostridium perfringens Type D In sheep and goats, sudden death without clinical signs being observed or short course neurological signs are strongly suggestive, although not specific, of acute *C. perfringens* type D enterotoxemia (Lewis, 2000; Uzal, 2004a). Neurological signs that last for 24 h to several days without other clinical signs are suggestive of subacute or chronic type D enterotoxemia in sheep (Uzal, 2004a). In goats, neurological signs with hemorrhagic diarrhea are suggestive of acute enterotoxemia, while hemorrhagic diarrhea that can last for several days is suggestive of the chronic form of the disease (Blackwell and Butler, 1992). Because recent evidence (Uzal et al., 2002b) suggests that *C. perfringens* type D can cause neurological disease in cattle, animals with acute neurological disease, in the absence of any other clinical signs, should also be investigated for type D enterotoxemia.

Clostridium perfringens Type E Because very little information is available about *C. perfringens* type E infection in most animal species, no presumptive diagnosis of infection by this microorganism can be established on the basis of clinical signs. An exception to this statement is the rabbit, in which enterotoxemia by *C. perfringens* type E is a well-defined entity in which sudden death of well-nourished animals or chronic disease with diarrhea, dehydration, and loss of appetite are clinical signs suggestive of this condition (Rehg and Pakes, 1982; Percy and Barthold, 2001).

Necropsy Findings

Clostridium perfringens Type A In sheep, generalized icterus, enlarged, pale and friable livers and red urine in the urinary bladder are characteristic, although not specific, of type A enterotoxemia (McGowan et al., 1958). However, *C. perfringens* type A enterotoxemia of sheep is a rare disease and other conditions, such as copper toxicity, should be considered before and in the list of differential diagnosis.

In goats, pathology of disease by *C. perfringens* type A is not well documented and the lesions described are not specific for this condition.

In cattle, hemorrhagic enteritis in young calves has been suggested as being associated with *C. perfringens* type A infection (Songer, 1996). However, since the role of this microorganism in disease of cattle has not been finally established, postmortem findings are of little value for the diagnosis of this condition. Cases of hemorrhagic enteritis in which other etiological agents have been ruled out should, anyway, be investigated for *C. perfringens* type A.

In chickens, necrotic enteritis with hemorrhagic sluggish intestinal content, associated or not with cholangiohepatitis, is highly suggestive of *C. perfringens* types A necrotic enteritis (Ficken et al., 1997).

Clostridium perfringens Types B and C Hemorrhagic enteritis in young lambs is suggestive of *C. perfringens* type B infection, while similar findings in adult sheep are suggestive of struck by *C. perfringens* type C (Lewis, 2000; Uzal, 2004a). Also, in older animals, hemorrhagic enteritis and focal symmetrical encephalomalacia (FSE; see below) are pathognomonic changes of *C. perfringens* type B infection (Lewis, 2000).

In goats, pathology of diseases by *C. perfringens* types B and C are not well documented, and the lesions described are not specific for these conditions (Barron, 1942, Gkiourtzidis et al., 2001; Uzal, 2004a).

In calves a few days old, *C. perfringens* type C produces necrohemorrhagic enteritis and these changes are characteristic but not specific for the condition (Barker et al., 1993).

In young foals with diffuse acute hemorrhagic enteritis in the jejunum and ileum, *C. perfringens* type C infection should be included in the list of differential diagnosis. These changes are, however, not specific for the condition (Howard-Martin et al., 1986).

In young (1–2 day-old) piglets, necrotic hemorrhagic enteritis is highly suggestive of *C. perfringens* type C enterotoxemia (Songer, 1996). In older animals (1–2 weeks of age), jejunal mucosa necrosis with nonbloody diarrhea is characteristic, although not specific for type C infection (Songer, 1996).

Clostridium perfringens Type D In sheep, FSE, when present, is pathognomonic and diagnostic for type D enterotoxemia (Hartley, 1956; Hornitzky and Glastonbury, 1993). However, this lesion is very rarely seen (F. A. Uzal, unpublished observation). Cerebellar coning as a consequence of brain edema is more frequently seen in cases of this disease and, although not specific for enterotoxemia, the presence of this lesion is highly suggestive of *C. perfringens* type D enterotoxemia (Uzal, 2004a). Other changes such as excess pericardial and/or pleural fluid and lung edema are suggestive but by no means specific of this disease. Gross changes may be entirely absent in a few cases of sheep enterotoxemia by *C. perfringens* type D (Barker et al., 1993; Uzal et al., 2004b). A negative necropsy should not, therefore, rule out enterotoxemia, and this disease should be included in the list of differential diagnosis for sudden death in sheep without gross postmortem findings. The so-called "pulpy kidney" lesion is considered a postmortem phenomenon (Barker et al., 1993; Uzal et al., 2004b) and has therefore little, if any, diagnostic significance.

In enterotoxemia by *C. perfringens* type D in goats, there may be changes that are suggestive of the disease, but none of these alterations is pathognomonic of enterotoxemia (Uzal, 2004a). Among these are excess pericardial fluid and lung edema in peracute and acute cases of the condition and fibrino-necrotic colitis in acute, subacute and chronic cases. Gross changes may be entirely absent in a few cases of caprine enterotoxemia by *C. perfringens* type D (Blackwell and Butler, 1992), and negative necropsy in animals with sudden death should not be used to rule out a diagnosis of enterotoxemia in goats.

Ancillary Tests There are a few diagnostic ancillary tests that are specific for *Clostridium perfringens* enterotoxemias. Among these are glucose detection in urine of sheep and goats with *C. perfringens* type D enterotoxemia. The presence of this metabolite in urine of these animals is usually interpreted as strongly suggestive of enterotoxemia. However, approximately 50% of the animals with type D enterotoxemia have no glycosuria (Uzal et al., 2004b) and although glycosuria is a useful indicator of the possibility of type D enterotoxemia, the converse does not apply, i.e., absence of glycosuria does not rule out a diagnosis of enterotoxemia in either species.

Other ancillary tests, such as observation of Gram-stained smears of intestinal mucosa can also be used to establish a presumptive diagnosis of enterotoxemia by any of the types of *C. perfringens* in most animal species. To be considered as a positive indicator of enterotoxemia, the Gram smears should show a large amount of Gram-positive rods with rounded ends, and although other bacterial forms can be seen, there should be a preponderance of rods (Carter, 1984; Fig. 32). Although it also has presumptive diagnostic value when it is positive, as with the case of glycosuria, Gram-stained smears of the intestinal mucosa cannot be used to rule out a diagnosis of enterotoxemia if they are negative.
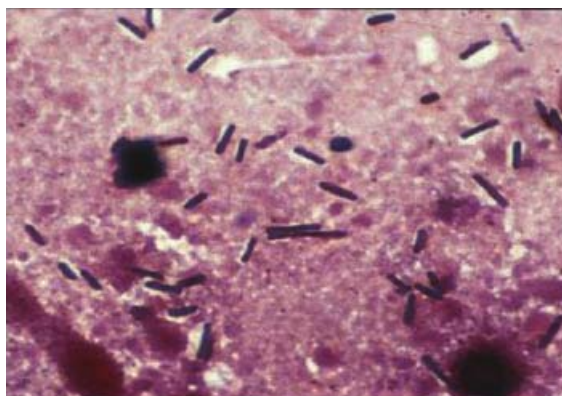
Fig. 32. Gram-stained smear of the small intestine mucosa of a goat with *C. perfringens* type D enterotoxemia.

Histopathology

Clostridium perfringens Types A, B and C The histopathological changes of infections by *C. perfringens* types A, B and C in most animal species are not specific and therefore do not have specific diagnostic significance, although they can be suggestive of these diseases.

Clostridium perfringens Type D In sheep, the histopathological changes produced by *C. perfringens* type D toxin in the brain (i.e., microangiopathy and/or focal symmetrical necrosis) are unique and pathognomonic for this type of enterotoxemia (Hornitzky and Glastonbury, 1993). These changes are valuable diagnostic criteria for diagnosis of type D enterotoxemia, particularly when results of toxin investigation in intestinal contents are not available.

It is unclear how frequently sheep dying from enterotoxemia fail to develop microangiopathy, although cases of enterotoxemia in which this lesion has not been seen are occasionally diagnosed (Uzal et al., 2004b). While cerebral microangiopathy is a useful indicator of enterotoxemia, the absence of this lesion does not preclude a diagnosis of this disease in sheep.

Although changes in the brain are not considered to be a common feature of caprine enterotoxemia by *C. perfringens* type D, microangiopathy and symmetrical degeneration of the brain may be observed in peracute and acute cases (Barker et al., 1993; Uzal et al., 1997b).

Histological lesions in the kidneys are not a characteristic of sheep, goat or cattle enterotoxemia, as the changes frequently described in cases of this disease are believed to be postmortem changes due to rapid autolysis of the tissues (Barker et al., 1993).

Clostridium perfringens Type E Except for the rabbit, descriptions of histological lesions by *C. perfringens* type E in animals are scant. In rabbits the histological changes of infection by *C. perfringens* type E are not specific for the condition (Percy and Barthold, 2001), although they may be used to establish a presumptive diagnosis of the condition.

Culture and/or PCR Detection Because all *C. perfringens* types can be normal inhabitants of the intestine of most animals, the mere culture of this microorganism from intestinal contents of animals is not diagnostic for infections by any type of this microorganism. However, quantitation of *C. perfringens* in intestinal content is considered an indicator of disease occurrence in animals (Wierup and DiPietro, 1981; Dart, 1988; Ohnuna et al., 1992) when large amounts of

C. perfringens, usually above 10^7 colony forming units (CFU)/g of intestinal contents or feces, are found. This appears true for *C. perfringens* type A and D enterotoxemias of lambs, in which elevated counts of *C. perfringens* are found in intestinal contents (usually, 10^4 to 10^7 CFU/g) and for cases of *C. perfringens* type C infection in piglets from which as many as 10^9 CFU of *C. perfringens* types A or C were isolated (Popoff, 1984; Ohnuna et al., 1992; Songer, 1996). However, high numbers of *C. perfringens* sometimes occur in the normal gut (Songer, 1996), and information about normal counts and their variations in most animal species is scant.

In sheep, while most authors seem to agree that the isolation of types A and D is of no diagnostic value, some researchers still believe that the isolation of types B or C is in itself of diagnostic value (Sterne and Batty, 1975). However, this latter belief is undermined by the fact that *C. perfringens* types B and C have been found in the intestinal content of healthy sheep (Itodo, 1986; Uzal and Marcellino, 2002a). More studies to assess the amounts of the different toxinotypes of *C. perfringens* in intestinal contents and feces of normal animals are necessary to clarify this point.

Several recent studies used PCR assays in attempts to correlate the intestinal presence of *C. perfringens* carrying different toxin genes with disease (Garmory et al., 2000; Gkiourtzidis et al., 2001). This approach is particularly useful in the case of toxins, such as β2, for which no detection assays are currently available. More information about the occurrence of the different toxin genes in isolates from healthy animals is necessary, however, to determine whether these genes correlate with disease.

Toxin Detection The most accepted criterion in establishing a definitive diagnosis of disease by *C. perfringens* in several animal species is the detection of its toxins in intestinal contents (Sterne and Batty, 1975). However, some of the major toxins of *C. perfringens* (e.g., α toxin) can also be found, albeit in small amounts, in the small intestine of clinically healthy sheep, goats and other animal species (Bullen, 1952). This poses a diagnostic challenge, because to the best of our knowledge there is no information about the levels of the toxin normally present in the intestine of clinically normal animals vs. diseased animals.

Probably one of the greatest impediments to the detection of *C. perfringens* toxins in body fluids is that very few diagnostic laboratories around the world perform toxin detection routinely. Furthermore, only a few commercial diagnostic systems are available, and those are for detection of only a few of the major *C. perfrin-*

gens toxins. Antibodies for the traditionally used mouse neutralization test are no longer commercially available.

Minimal standardization exists between different laboratories or techniques for *C. perfringens* detection. This is evident from a recent evaluation and comparison of four techniques used by four different diagnostic laboratories to detect *C. perfringens* type D toxin in intestinal contents and other body fluids of sheep and goats (Uzal et al., 2003). This study showed a marked inconsistency among the four techniques employed, suggesting that until more information is available about the reliability of these techniques, the diagnosis of *C. perfringens* infections should not be based solely on toxin detection but also on consideration of clinical and pathological data.

While detection of and β toxins (by any of the methods currently available) in intestinal contents of animals suspected to have died of enterotoxemia is considered diagnostic for this disease, failure to detect these toxins should not exclude a diagnosis of enterotoxemia. This is particularly true for β toxin, which is very sensitive to the action of trypsin and can be destroyed after a few hours presence in the intestine (Sterne and Batty, 1975). Epsilon toxin is a much more robust toxin that can remain active for up to 48 weeks at 4°C (Bennett, 1961). However, failure to detect this toxin in intestinal contents of sheep does not rule out a diagnosis of enterotoxemia if the pathological changes (i.e., microangiopathy and/or focal symmetrical degeneration of the brain) are present (Buxton et al., 1978b).

PREVENTION Prevention of most *C. perfringens* enteric infections of animals is based mainly on nutritional management (Bullen, 1952) and vaccination (Kennedy et al., 1977; Odendaal et al., 1989; Lewis, 2000). The latter applies only to those conditions for which vaccines are commercially available.

As would be expected, prevention is best understood for those conditions in which the epidemiology and pathogenesis are better known. This is the case for type D infections in sheep and goats and type C infection in piglets.

Nutritional management to prevent cases of type D enterotoxemia in sheep and goats mainly involves avoiding sudden changes in diet, particularly from poor rations to diets rich in highly fermentable carbohydrates (Bullen, 1952). A minimum of two weeks with daily progressive increase of the new diet should be allowed for the ruminal microflora to adapt to the new ration (Merchen, 1988).

Most clostridial commercial vaccines are multivalent, including antigens from several clostrid-ial species in addition to *C. perfringens* antigens. The *C. perfringens* components of these vaccines typically consist of inactivated cells, toxins or both (Hjerpe, 1990; Songer, 1996). The immunogenic value of the *C. perfringens* bacterial cells is, however, doubtful, and the potency control of the *C. perfringens* vaccines is based on the ability of the toxoids to generate antibodies in laboratory animals (Anonymous, 1993).

Currently, vaccines containing α, β and toxoid are commercially available. These vaccines are used regularly in most countries of the world in sheep. In this animal, vaccination with vaccines containing β and toxoid drastically reduces the occurrence of disease by types B, C and D of *C. perfringens*. Two initial doses of vaccine are recommended when lambs are 4–6 months of age, followed by an annual booster 4–6 weeks before lambing (Lewis, 2000), which confers protective levels of colostrum antibodies to the lambs until their first dose of vaccine (Bullen, 1952; Kennedy et al., 1977; Odendaal et al., 1989; Lewis, 2000).

Because no vaccines are manufactured specifically to prevent enterotoxemia in goats, vaccines developed for sheep are generally used in this species (Blackwell et al., 1983). However, in goats these vaccines elicit antibody titers of lower magnitude and shorter duration than in sheep, and although they appear to reduce the incidence and severity of caprine enterotoxemia (Shanks, 1949), these vaccines do not seem to prevent the occurrence of enterocolitis in goats (Shanks, 1949; Oxer, 1956; Guss, 1977; Blackwell et al., 1983). The reason for the difference in the responses of sheep and goats to vaccination with toxoid is not well understood, but it has been suggested this is due to the action of toxin locally in the colon of goats, an environment where serum antibodies produced in response to parenteral vaccination would not be very efficient (Uzal and Kelly, 1996). It has been demonstrated, however, that very high titers of toxin antibodies obtained after experimental parenteral vaccination of goats with oil adjuvanted toxoid vaccines, protect goats against both the systemic and colonic effects of experimental type D enterotoxemia (Uzal and Kelly, 1998a). Blackwell et al. (1983) and Uzal et al. (1998b) recommended that to maintain antitoxin titers at protective levels in goats, it is necessary to vaccinate the animals three to four times a year. Initial vaccination should be followed by a booster 3–6 weeks later, and pregnant females must receive the last vaccination 2–3 weeks before parturition. Kids should receive their first dose at 4–6 weeks of age (Smith and Sherman, 1994).

There are also commercially available vaccines to protect piglets against necrotic enteritis by vaccination of the dams during gestation

(Ripley et al., 1983). Vaccination with these products has reportedly achieved an up to tenfold reduction in mortality (Udovicic et al., 1990).

Although commercial vaccines including α toxoid, in addition to several other toxoids, are available in several countries; no information has been published about the protective effect that these vaccines might have on intestinal or muscle disease caused by *C. perfringens* type A.

It is believed that vaccination with CPE toxoid reduces enteric disease in pigs (Estrada Correa and Taylor, 1989), although to the best of our knowledge no CPE vaccines are commercially available.

Applications

Recent studies suggest the exciting possibility of using CPE as a therapeutic agent against certain solid tumors. One study found that claudin-4, which can serve as a functional CPE receptor, is highly expressed in most pancreatic cancer cells (Michl et al., 2001). This study then demonstrated that direct injection of CPE into pancreatic tumor xenografts (growing in mice) induces necrosis and shrinkage of those tumors.

The promising antitumor activity of CPE is not limited to pancreatic cancer. Overexpression of claudin-3, another functional CPE receptor, has been detected in prostate adenocarcinomas, and these tumor cells were also found to be highly CPE-sensitive (Long et al., 2001).

Clostridium difficile

Clostridium difficile, a sporeforming, Gram-positive anaerobe is an example of an opportunistic pathogen that takes advantage of people with compromised intestinal flora. When the normal intestinal microflora is altered by antibiotics, *C. difficile* can become established and produce its tissue-damaging toxins, resulting in the onset of disease. Nearly all antibiotics have been associated with the disease, but ampicillin, amoxicillin, clindamycin, and cephalosporins are the most common. The resulting infection can range from asymptomatic carriage, to diarrhea, to life-threatening pseudomembranous colitis. Fortunately, new and improved diagnostics are now available so that the disease can be identified before it develops into the severe stages. The general terms used to describe *C. difficile* disease include antibiotic-associated diarrhea (AAD), antibiotic-associated colitis (AAC), and pseudomembranous colitis (PMC). AAD is the milder form of the disease, and in some instances, is self-limiting. AAC and PMC have a strong inflammatory component and may become life-threatening if not accurately diagnosed and treated promptly.

Clostridium difficile causes almost all cases of pseudomembranous colitis, although in a few instances *Staphylococcus aureus* has been suggested by some to cause PMC. The involvement of *S. aureus* was proposed in the 1950s and 1960s prior to the discovery of *C. difficile* as an intestinal pathogen, and many doubt that *S. aureus* was ever involved. However, there are rare instances in which *C. difficile* cannot be cultured or identified by other methods (e.g., detection of toxin by tissue culture or antibody-based tests, or detection of toxigenic strains by PCR) from the patient's stools, indicating that other causes of PMC are rare. *Clostridium difficile* is the most recognized cause of AAD, but this organism causes only 25% or less of these cases. More than 75% of AADs remain undiagnosed.

Because of improved diagnostics, *C. difficile* disease is recognized more promptly, reducing the number of cases that progress to PMC. Current antibiotic regimens used to treat this antibiotic-initiated disease are effective in most cases. However, relapses continue to be a major problem, and the incidence of *C. difficile* disease (primarily AAD) continues to occur at a constant rate. Recent estimates indicate that about 300,000 *C. difficile* cases occur annually in the United States. From an economic perspective, the disease costs roughly $5,000–10,000 per case, with higher costs incurred for relapsing patients. When the costs of diagnosis, therapy, and extended hospital stay are considered, this disease adds more than a billion dollars annually to healthcare costs in the United States.

Historical Perspectives

Clostridium difficile was first described by Hall and O'Toole in 1935 (Hall and O'Toole, 1935). The original isolates, named "*Bacillus difficilis*," because of the difficulty in isolating the organism, were obtained from stool specimens from healthy infants. Although believed to be harmless members of the microflora, the investigators showed the isolates were toxigenic in guinea pigs. The role of this organism in human disease went unnoticed until the late 1970s. Some cases of PMC attributed to *S. aureus* were reported throughout the 1950s and 1960s, but the organism could not be isolated consistently from stool specimens obtained from PMC patients. In addition, *S. aureus* was present in stool specimens from many apparently healthy persons. Since these reports, no additional studies appeared supporting a primary role for *S. aureus* in the onset of PMC.

The importance of PMC as a complication of antibiotic therapy became apparent through the

results of studies done in the mid-1970s. A landmark study by Tedesco et al. (1974) reported a PMC incidence of 10% in patients receiving clindamycin, resulting in the phrase "clindamycin-associated colitis." Within 2–3 years, researchers described the presence of a "cell-rounding" activity in stool specimens from persons with antibiotic-associated PMC (Bartlett et al., 1977; Bartlett et al., 1978; Larson et al., 1977; Rifkin et al., 1977). The rounding of cells was neutralizable by gas gangrene antisera and, more specifically, by *C. sordellii* antisera. However, investigators were unable to recover *C. sordellii* in stool specimens from PMC patients. Further analysis soon led to the discovery of *C. difficile* in the specimens. The role of this organism as the cause of antibiotic-associated diarrhea and colitis was established by a series of studies showing that 1) human isolates of *C. difficile* isolated from PMC patients could be grown in vitro, 2) culture filtrates from these isolates caused the same type of cell rounding as that observed directly in the stool specimens, 3) the activity was neutralized by *C. sordellii* antisera, and 4) hamsters challenged with human PMC isolates developed severe intestinal disease and died. The puzzling cross-neutralization by *C. sordellii* antisera was explained shortly thereafter by results showing that *C. sordellii* produces two toxins immunologically related to those produced by *C. difficile*. Antiserum against the *C. sordellii* toxins cross-reacted and cross-neutralized the toxins from *C. difficile*, resulting in the initial confusion surrounding any role of *C. sordellii* in the disease.

Until the late 1970s, only a single toxin had been discovered. In 1980, Dr. John Bartlett and his research team in the United States (Taylor et al., 1980) and Dr. Ueno's research team in Japan (Banno et al., 1981) identified a second toxin. The first toxin discovered, toxin B, is also referred to as the "cytotoxin." The second toxin, toxin A, often is referred to as the "enterotoxin." The toxins are named "A" and "B" on the basis of their order of elution during anion-exchange chromatography on resins such as diethyl amino ethyl (DEAE) Sepharose and Sephadex. Toxin A binds more weakly owing to its lower negative charge than toxin B and elutes from anion-exchange resins with lower levels of NaCl than toxin B. Therefore, it elutes prior to toxin B. Both toxins are cytotoxic, but toxin B is much more active against most mammalian cells in tissue culture assays than toxin A. Toxin A, on the other hand, is a tissue-damaging enterotoxin that is much more active in the intestinal tract than toxin B in various animal models. Both toxins are lethal when injected systemically, and they appear to act synergistically in the intestine.

Taxonomy and Phylogeny

On the basis of 16S ribosomal RNA sequencing, *C. difficile* is included in cluster XIa of the clostridia. With the exception of *Clostridium sordelli*, that cluster contains no other pathogenic species.

Phenotypically, *C. difficile* closely resembles *C. sporogenes*, although the two species can be differentiated by metabolic properties. The mol% G+C content is 28%. A collaborative effort between researchers in London and the Sanger Institute has led to the sequencing of a toxigenic isolate (*C. difficile* strain 630 type X). The genome is a circular chromosome of 4,290,252 bp with a G+C content of 29.06%. There also is a circular plasmid of 7,881 bp with a G+C content of 28.9% in this strain Sanger Sequencing Center (http://www.sanger.ac.uk/Projects/C_difficile/).

Habitat

Clostridium difficile only thrives in the adult intestinal tract when the normal flora has been altered or compromised, usually by antibiotics. Other drugs such as antineoplastic agents also alter the intestinal flora, predisposing patients to infection with this pathogen. In healthy persons, the intestinal carriage rate is 3% or less, with slightly higher rates reported in Japan (Mulligan, 1988). In contrast to its low carriage rate in adults, *C. difficile* is present in a high number of infants and is (or should be) considered a member of the normal microflora. The organism and its toxins can be detected in a high percentage of infants (in some studies, 50% or higher). The level of colony-forming units and toxin in feces from completely healthy infants may be comparable to the levels observed in feces from adults with severe *C. difficile* disease. It is not understood why there is this difference in susceptibility to disease between infants and adults. The organism persists in the environment in the spore stage, making it extremely difficult to eradicate. In the rooms of *C. difficile* patients, the organism can be isolated from the linens, curtains, books, walls and floor surfaces.

Most studies of this organism have focused on its presence in the intestinal tract where it causes disease. However, *C. difficile* also has been isolated from the genitourinary tract (Hafiz and Oakley, 1975). It is suspected that infants tend to become colonized with the organism through environmental sources.

Clostridium difficile has been isolated from a wide variety of animals. Many animals (horses, camels, cows, donkeys, rabbits, household pets such as dogs, cats and birds) carry low numbers of the organism but do not become ill. Some

animals, however, do develop intestinal illness similar to that observed in humans. Hamsters are extremely sensitive to this organism and its toxins, and develop an acute onset of intestinal disease manifested primarily as a fatal cecitis. For this reason, hamsters are the animal model of choice and have proved valuable for learning more about the pathogenesis and therapeutic aspects of the disease. Antibiotic-treated mice also can develop the disease, but in mice, the disease develops more as a chronic colitis. For this reason, some researchers believe the mouse model more closely mimics the onset of disease in humans. Horses and pigs treated with antibiotics also can develop *C. difficile* intestinal disease.

Isolation

Clostridium difficile requires anaerobic conditions for growth. However, it is not as strict an anaerobe as some of the other clostridia (e.g., *C. tetani*). Cycloserine-cefoxitin-fructose agar (CCFA), originally developed by George et al. (1979), continues to be the most commonly used selective and differential plate media for culturing the organism from stool specimens. The original formulation consists of egg yolk-fructose agar base (Difco Laboratories, Detroit, MI) supplemented with cycloserine (500 µg/ml, final concentration), cefoxitin (16 µg/ml, final concentration), and 5% egg yolk suspension. CCFA is highly selective, but when culturing fecal specimens, some Gram-negative anaerobes, lactobacilli, and other clostridia will grow on the agar. The inclusion of horse blood improves the recovery of the organism (Marler et al., 1992). Other modifications that reportedly improve the recovery of the organism from fecal specimens and environmental sources include 0.1% sodium taurocholate in place of the egg yolk suspension, decreasing the cycloserine and cefoxitin concentrations in half, the addition of cysteine hydrochloride as a growth supplement, and norfloxacin (12 µg/ml, final concentration) and moxalactam (32 µg/ml, final concentration) as the selective agents (Levett, 1984; Bartley and Dowell, 1991; Aspinall and Hutchinson, 1992). On solid media, *C. difficile* gives flat rough colonies that grow to several millimeters in diameter. Under long-wavelength ultraviolet light, the colonies will fluoresce. The intensity of the fluorescence can be increased by the addition of blood or brain-heart infusion, and nonspecific fluorescence may be reduced by the addition of blood to CCFA.

Selective broths may also be used to recover *C. difficile* from stool specimens. Cycloserine-cefoxitin-fructose broth can be prepared using the original formulation of George et al. but omitting the agar. In our laboratory, we routinely use brain-heart infusion broth supplemented with cycloserine (500 µg/ml) and cefoxitin (16 µg/ml). Broth cultures are more easily and efficiently analyzed for *C. difficile* toxin by ELISA or tissue culture than colonies isolated on solid media.

Identification

Clostridium difficile is a Gram-positive anaerobe that produces subterminal spores. The vegetative cells may be motile in broth cultures with peritrichous flagella. The sporulating ability of *C. difficile* varies from strain to strain. Unlike the enterotoxin of *C. perfringens*, spore production of *C. difficile* is not correlated with toxin production. The ability of this organism to form spores is the primary reason that *C. difficile* is such a problem in medical facilities. Laboratories that utilize bacterial culture often use heat-shock procedures or the incorporation of sodium thioglycollate and lysozyme into the isolation medium to improve the recovery of spores from specimens (Wilson, 1983; Clabots et al., 1989; Kamiya et al., 1989). In some strains, capsules and fimbriae may be present. However, these have only been described in a few articles, and their role in the virulence of this organism has not been well characterized (Borriello et al., 1988; Strelau et al., 1989; Davies and Borriello, 1990; Baldassarri et al., 1991). As with spore formation, there is no correlation of capsule or fimbriae production with toxin production.

This organism produces a number of antigens that crossreact with antigens from other clostridia and anaerobes. In addition to the crossreaction of toxins A and B with *C. sordellii* hemorrhagic toxin (toxin HT) and *C. sordellii* lethal toxin (toxin LT), respectively, there are other crossreactive antigens. The cell wall consists of array proteins that are related to cell wall antigens from *C. sordellii* and *C. bifermentans*. There are extractable antigens, including a 36-kDa protein that has been shown to elicit an antibody response in patients with *C. difficile* disease. No function has been assigned to this particular antigen (Poxton and Cartmill, 1982; Masuda et al., 1989; Pantosti et al., 1989; Cerquetti et al., 1992). Glutamate dehydrogenase, also referred to as the "common antigen," crossreacts with the glutamate dehydrogenase from *C. sporogenes*, proteolytic *C. botulinum*, and *Peptostreptococcus anaerobius*. The *C. difficile* enolase is immunologically related to *C. bifermentans* enolase.

Preservation

Anaerobic cultures of *C. difficile* can be lyophilized, and freeze-dried material is stable for

years. The organism also can be preserved for years as spores in cooked meat media. Screw-capped or stoppered tubes of the media can be inoculated with strains and incubated for 24–36 h at 37°C or until there is obvious growth. The tubes can then be stored on the shelf at room temperature with the cultures remaining viable for years.

Physiology

Clostridium difficile needs anaerobic conditions for growth. The organism is nonhemolytic, egg yolk negative, and weakly or nonproteolytic under typical culture conditions. Glucose is utilized as a carbon source, but other sugars, including sucrose, lactose and maltose, are not suitable carbon sources. *Clostridium difficile* produces acetic, isobutyric, butyric, isovaleric, valeric and isocaproic acid through fermentation. The most unusual of these is isocaproic acid (Johnson et al., 1989). Isocaproic acid has been suggested as a marker for the organism, but this fermentative product also is produced by *C. bifermentans*, *C. sordellii* and *C. sporogenes*. Another unusual product is *p*-cresol, which is produced through the metabolism of tyrosine (Levett, 1987; Sivsammye and Sims, 1990). *Clostridium scatologenes*, which is seldom encountered in clinical specimens, is the only other *Clostridium* species known to produce *p*-cresol. Like isocaproic acid, *p*-cresol has been suggested as a marker for *C. difficile* in clinical specimens. However, *p*-cresol has not been utilized because it requires expensive equipment, and its detection does not distinguish toxigenic from nontoxigenic isolates.

Clostridium difficile utilizes *N*-acetyl-glucosamine and *N*-acetyl-neuraminic acid and other substrates through the production of hydrolytic enzymes that include collagenase, some protease, and mucopolysaccharide-hydrolyzing enzymes. In addition, the organism produces arylesterase, sialidase, sialate *O*-acetylesterase, *N*-acetylneuraminate lyase and glycosulfatase (Corfield et al., 1992). These activities allow *C. difficile* to utilize mucin components as its main energy source. These enzymes are produced by many members of the intestinal microflora and are not unique to *C. difficile*.

Toxins

TOXIN GENETICS Both of the genes for toxins A and B have been cloned and sequenced (Von Eichel-Streiber et al., 1988; Von Eichel-Streiber et al., 1992a, 1992b; Dove et al., 1990; Johnson et al., 1990; Barroso et al., 1990). There are five ORFs in the pathogenicity islet encoding toxins A and B (Fig. 33). The *toxB* gene is approximately 1 Kb upstream of the *toxA* gene. Upstream of the *toxB* gene is an ORF (*txeR*) that positively controls regulation of *toxA* and *toxB*. There is a small reading frame located between *toxA* and *toxB* that encodes a potential lipoprotein signal sequence at its N-terminus. The small ORF designated "txe3" is transcribed in the opposite direction of the other genes in the pathogenicity islet. There has been speculation that *txe3* may play a role in negative regulation of *toxA* and *toxB*, but this remains to be determined. None of the small ORFs are involved in the biological activity of either toxin A or toxin B. Recombinant toxin A and toxin B have been expressed from *toxA* and *toxB* cloned independently of the other ORFs. Recombinant toxin A is enterotoxic, cytotoxic, and lethal, and agglutinates rabbit erythrocytes whereas recombinant toxin B is cytotoxic and lethal. Nontoxigenic strains of *C. difficile*, which comprise roughly 20% of the clinical isolates, do not carry any of the ORFs in the pathogenicity islet. Instead, they contain a 127-bp sequence at the locus where the pathogenicity islet is located. This sequence is not present in toxigenic strains. There are inverted repeats with predicted the secondary structure in the sequence, suggesting that this may be an insertion site.

The distance between the transcriptional start site and the ATG start codon is 169 nucleotides for *toxA* and 239 nucleotides for *toxB* (Von Eichel-Streiber et al., 1995; Hammond et al., 1996; Hundsberger et al., 1997; Dupuy and Sonenshein, 1998; Song and Faust, 1998). Under in vitro growth conditions, *C. difficile* produces higher levels of toxins A and B when grown slowly. Other clostridial toxins (e.g., botulinum neurotoxin) also exhibit the property of higher expression during slow growth. Rapid growth, defined media, and glucose all affect toxin production, resulting in lower levels of toxins A and B (Dupuy and Sonenshein, 1998).
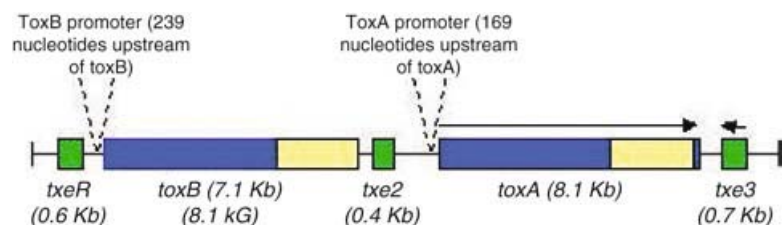
Fig. 33. The pathogenicity islet encoding toxins A and B.

The *txeR* element located upstream of *toxB* encodes a small molecular weight protein (Mr 22,000) of 184 amino acids. This protein contains an unusually high number of lysine residues within the C-terminus. Further analysis through protein data libraries (Moncrief et al., 1997) shows the presence of a helix-turn-helix motif with sequence identity to DNA binding bacterial response regulators, including UviA, a positive regulator of bacteriocin production in *C. perfringens* (Garnier and Cole, 1988a; Garnier and Cole, 1988b), *botR* of *C. botulinum*, a positive regulator of botulinum neurotoxin C1 (Marvaud et al., 1998a, 1998b), and *tetR*, a positive regulator of the *C. tetani* tetanus toxin gene (Marvaud et al., 1998b). The putative role of the *txeR* element as a positive regulator is demonstrated by studies showing that when expressed in *trans* with the *toxB* promoter linked to the repeating units of toxin A (designated "toxin A repeating units" [ARU]), the expression of ARU increases 800-fold. Further, when the toxin A promoter is fused to ARU and *txeR* is supplied in *trans*, the expression increases over 500-fold (Moncrief et al., 1997). Some early initial studies on the transcription of *toxA* and *toxB* suggested the possibility that the toxins are transcribed as part of a large polycistronic mRNA and that smaller mRNAs possibly result from post-translational processing (Hammond et al., 1996). However, more recent studies indicate that most of the transcription occurs from the individual toxin gene promoters, and that the *txeR* gene product is involved.

It has been suggested that *txeR* may be a σ factor that turns on toxin production in response to environmental conditions such as limited nutrients (e.g., the conditions that exist in brain heart infusion dialysis sac cultures). In fact, *txeR* actually appears to stimulate its own synthesis (Mani et al., 2002). High-level transcription would then occur as the levels of the *txeR* product increase (Moncrief et al., 2000a, 2000b). This proposed mechanism is supported by recent studies showing that *txeR* encodes a positive regulator that allows an RNA polymerase to recognize the promoters of *toxA* and *toxB*. In addition, *txeR* directly stimulates its own synthesis. These findings support the idea that during rapid growth, *txeR* is repressed and little production of either toxin A or toxin B occurs. During slower growth (e.g., during stationary growth), the repression of *txeR* decreases, resulting in stimulation of *txeR*, and in turn increased transcription of *toxA* and *toxB*.

TOXIN STRUCTURE Toxins A and B are produced as large single polypeptide chains that are released following autolysis as the organism enters stationary phase growth. Neither toxin contains a signal sequence or requires proteolytic activation. Examination of the primary structure of toxins A and B shows that the two toxins are highly related in their basic structure (Fig. 34). This similarity is especially interesting since the two toxins demonstrate no significant immunological crossreaction, and antitoxin A and B antibodies do not cross-neutralize the heterologous toxin (Libby and Wilkins, 1982; Lyerly et al., 1986). The toxins have an overall homology of >45% at the amino acid level. Structurally, both contain a series of contiguous repeating units at the COOH terminus, comprising roughly one-third of the molecule (Price et al., 1987; Lyerly et al., 1989; Von Eichel-Streiber and Sauerborn, 1990; Von Eichel-Streiber et al., 1992b). The repeating portions, which are comprised of large repeating units made up of smaller repeating units, have a homology of approximately 41%. The repeating units of toxin A comprise the temperature-dependent hemagglutinating activity of the molecule. The enzymatic site in each toxin is located upstream near the N-terminus. Several other features are conserved, including four cysteine residues located in almost identical positions, a putative ATP binding region, and a hydrophobic membrane-spanning region located near the center of each toxin.
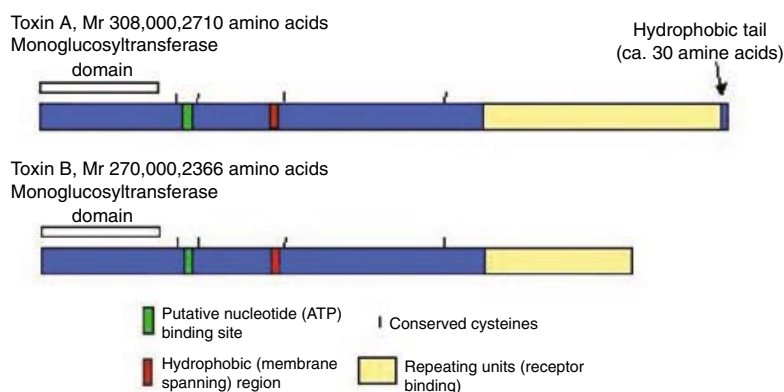
Fig. 34. Structure/function relationships for *C. difficile* toxins A and B.

The repeating units most likely represent the binding portion of the toxin. In toxin A, the repeating units bind to galactose-containing residues. Mammalian cells that carry Galα1-3Galβ1-4GlcNAc residues are much more sensitive to toxin A than cells that do not have these residues (Wilkins and Tucker, 1989; Tucker et al., 1990). For example, F9 teratocarcinoma mouse cells, which have the galactose-containing residues on their cell surface, are a hundred times more sensitive to toxin A than CHO cells, which lack the galactose residues. Toxin A also binds to human intestinal tissue, although the binding is not mediated through Galα1-3Galβ1-4GlcNAc residues, which are absent in the human intestine. Interestingly, however, toxin A binds to the I, X, and Y (Lewis X [Le^x] and Lewis Y[Le^y]) carbohydrate blood group antigens, all of which are galactose-containing moieties that contain the type 2 core Galα1-3Galβ1-4GlcNAc that binds toxin A (Tucker and Wilkins, 1991). Possible receptors for toxin B have not been identified. Both toxins probably can enter mammalian cells via nonspecific mechanisms such as pinocytosis.

MECHANISM OF ACTION As might be inferred from the high level of homology at the amino acid level, toxins A and B share enzymatic properties. Both are monoglucosyltransferases that covalently modify the cellular protein Rho (Just et al., 1994; Just et al., 1995a, 1995b, 1995c; Dillon et al., 1995). Rho is a member of the Rho/Rac family (RhoA, RhoB, RhoC, Rac 1, Rac 2, and CDC42) of low molecular weight guanosine 5' triphosphate (GTP)-binding proteins that are involved in the regulation of the cellular cytoskeletal system. Each toxin is capable of transferring glucose from uridine diphosphate glucose to Rho. When this occurs, the ability of Rho to be involved in the dynamic equilibrium of soluble to insoluble actin is impaired. The disruption of the cellular cytoskeleton most likely results in the cell rounding and eventual cell death observed with these toxins. Other members of the large clostridial cytotoxin group also share this ability, although C. sordellii toxin LT monoglucosylate members of the Ras subfamily and α-toxin from C. novyi use UDP-GlcNAc rather than UDP-Glc as the substrate (Von Eichel-Streiber et al., 1996). The glucosyltransferase domain resides within the first 546 amino acids of the toxins (Hofmann et al., 1997; Wagenknecht-Wiesner et al., 1997). The location of the domain has been demonstrated by the microinjection of a 63-kDa recombinant fragment of toxin B into NIH-3T3 cells, which caused the cells to round identically to cells treated with holotoxin. In addition to the inactivation of Rho, other possible mechanisms of toxin A may be its ability to damage mitochondria and activate other pathways such as p38 MAP kinase pathway. Toxin A also may increase the expression of certain cytokines (e.g, IL-8) independent of its effect on Rho (Liu et al., 2003).

ATYPICAL TOXIGENIC STRAINS Within the group of toxigenic C. difficile, there are several distinct toxin phenotypes: A+/B+, A–/B+, and A+/B–. Typical A+/B+ isolates are by far the most prevalent. Typical isolates produce toxin A and toxin B. The toxins appear to be coordinately regulated since strains produce either high, intermediate or low levels of each toxin. Within this group, strains may vary by more than 100,000-fold in the amount of toxin produced in vitro. Typical toxigenic isolates probably account for 95% or more of the cases of C. difficile disease. Atypical A–/B+ isolates, which have only recently been described, comprise 5% or less of the clinical isolates. Atypical isolates produce only toxin B. The A–/B+ isolates carry a full-length toxB gene but only a truncated toxA gene. In addition, the truncated toxA gene has a stop codon located close to the 5' end of the gene. Atypical A–/B+ isolates can cause disease and death even though they do not produce toxin A. Toxin B from atypical A–/B+ isolates crossreacts extensively with toxin B from typical strains. However, the atypical toxin B is approximately tenfold more cytotoxic and lethal. In addition, it mimics C. sordellii toxin LT in that it monoglucosylates a broader range of G-proteins, possibly explaining the increased toxicity. Only a single A+/B– isolate has been reported, and it was isolated from a horse.

IOTA TOXIN Some strains of C. difficile produce τ toxin, which is a binary ADP-ribosylating toxin that modifies actin (Perelle et al., 1997; Braun et al., 2000; Gulke, 2001). The toxin is composed of a 48-kDa enzymatic component (referred to as "CDTa") and a 94-kDa binding component (referred to as "CDTb"). These two components are not covalently linked. Within CDTa, the N-terminal portion interacts with CDTb, and the C-terminal portion carries out the enzymatic activity (Stubbs et al., 2000). The τ toxin is a member of the class of binary ADP-ribosylating toxins that include τ toxin from C. spiroforme and C. perfringens. The τ toxin from C. difficile, C. spiroforme and C. perfringens crossreact immunologically, both with the enzymatic component and the binding component. Also included in this group of binary ADP-ribosylating toxins is C2 toxin from C. botulinum. C2 toxin, however, does not crossreact immunologically with any of the τ toxins. Current estimates suggest that the number of strains that carry a functional τ toxin is roughly 12% or less. Epidemiological studies

are under way to determine the prevalence of clinical strains that produce τ toxin and whether there are A–/B– strains that carry the τ toxin gene. The τ toxin has only been associated with a few toxinotypes (Rupnik, 2002; see Epidemiology). Currently, the role of τ toxin in *C. difficile* pathogenesis is unknown.

Epidemiology

Epidemiology studies continue to be performed on *C. difficile* primarily because outbreaks are a major problem. The disease does not occur because of antibiotic-resistant isolates. *Clostridium difficile* is sensitive to the antibiotics that cause the disease. Rather, the organism begins to grow as the level of antibiotic in the intestine decreases.

Current estimates indicate that approximately 300,000 cases of *C. difficile* disease occur annually in the United States, and there is no indication that the incidence rates are decreasing. The disease is especially problematic in hospitals with large populations of elderly adults (e.g., Veterans Adminstration hospitals), but it has been reported at all levels of healthcare facilities. It is important that healthcare personnel become familiar with general knowledge about *C. difficile*, its role as a hospital-acquired pathogen, and the fact that it is spread most commonly by hospital personnel.

A variety of methods, including plasmid and bacteriocin typing, bacteriophage typing, electrophoretic profiles, and serotyping, have been used to study isolates from outbreaks. Bacteriophage, plasmid and bacteriocin typing are restrictive because only about 75% or less of the isolates can be typed. More recently, restriction endonuclease mapping has been used effectively to identify and group clinical isolates. All of these methods have provided evidence that outbreaks are due to in-house strains and that strains are spread effectively from patient-to-patient through contact with healthcare workers. Clinical isolates can be obtained not only from the patient, but also from linens, curtains and furniture in the infected patient's room. Spores of the isolate can persist for months to years, further complicating any efforts to decontaminate the room and minimize dissemination of the organism.

Asymptomatic carriage of toxigenic *C. difficile* occurs at a rate probably higher than most medical professionals realize. In one study, 21% of the patients who were negative when entering the hospital became positive while hospitalized (McFarland et al., 1990). The majority of these (63%) remained asymptomatic even though most received antibiotics. Patients that become asymptomatic carriers tend to have low or unde-tectable levels of toxin but high numbers of the organism. The reasons for this are unclear, but asymptomatic carriers can spread their isolates to other patients, who then develop *C. difficile* disease. In general, procedures for identifying carriers and limiting the risk factors associated with carriers are not implemented at most institutions. In most instances, any efforts to identify carriers are limited by costs and risk analysis. Carriers may be a primary risk factor for elderly patients who are transferred to long-term care facilities, and in some instances, transfers to these facilities have been denied because of asymptomatic carriage status.

Many infants (50% or higher in some studies) carry toxigenic *C. difficile* in their intestines but do not develop any signs of clinical illness. The resistance of infants to *C. difficile* disease has been recognized for some time (since the early 1980s), but there still is no clear understanding of the mechanism of protection. It appears that colonization by infants is due to environmental strains, and that isolates from infants can be spread through hospital wards. In addition, there have been several instances in which the isolates shed by the infant caused AAD in mothers treated with antibiotics after birth (McFarland et al., 1998). There also have been reports of infants who appear to develop a true case of *C. difficile* disease (Tvede et al., 1990; Kader et al., 1998). In these instances, the infants had some predisposing factors (premature birth, sepsis requiring antibiotics, etc.) that affected the integrity of the mucosa and mucosal microflora. Thus, on rare occasions, infants can develop the disease, but in general, *C. difficile* disease in infants is very uncommon.

Nontoxigenic strains form spores and are spread through hospitals similarly to toxigenic strains. On occasion, patients can be co-infected with multiple strains, including both a nontoxigenic and toxigenic strain. For this reason, it is important to confirm the presence of a toxigenic strain because nontoxigenic strains do not cause disease. Patients colonized with nontoxigenic strains do not need to be treated for *C. difficile* disease. In fact, nontoxigenic strains may be protective by competing with toxigenic strains for available nutrients (Borriello and Barclay, 1985b). For this reason, if bacterial culture is used as a diagnostic aid, multiple colonies should be selected and evaluated for toxin production.

The results of a number of studies based on serotyping suggest that certain strains of *C. difficile* are associated with particular target populations. For example, in some institutions, serogroups associated with elderly adults have been shown to be distinct from those associated with infants. This has been of interest because of the resistance of infants to *C. difficile* disease.

Several studies from the early 1980s indicate the association of a particular strain of *C. difficile* with cystic fibrosis patients in the absence of any disease. However, no follow up studies have appeared. In addition, a particular serogroup has been associated more closely with acquired immunodeficiency syndrome (AIDS) patients. In this instance, it was suggested that the strains, which were more resistant to tetracycline, chloramphenicol, erythromycin, rifampin, and clindamycin than other strains, were more closely associated with AIDS patients because these patients were on multiple antibiotic therapy. The interpretation of results describing the association of specific isolates and patient groups is complex. Many of these studies involved isolates from the same or close facilities, suggesting that in some instances, the close association of strain to patient group resulted from the spread of an in-house strain.

Most recently, efforts to characterize groups of A+/B+ and A–/B+ isolates have centered on toxinotyping utilizing PCR and restriction analyses of the pathogenicity locus. The characterization studies have been performed on *C. difficile* collections in Belgium, Wales, Japan, Korea and Indonesia (Rupnik et al., 1997; Rupnik et al., 1998; Rupnik et al., 2003). On the basis of distinct deletions in different portions of the *toxA* and *toxB* genes, as well as insertions in other portions of the pathogenicity locus, at least 20 toxinotypes (designated "toxinotypes I–XX") have been identified. The prevalence of these isolates varies from hospital to hospital and from country to country. Most of the isolates fall into the A+/B+ category, but within this group, there are variant toxinotypes. New findings suggest that the A–/B+ group, which originally appeared to be primarily a coherent group, is comprised of variant toxinotypes as well. Recently, a pathogenic clinical isolate was identified that produces a variant toxin B (with broad substrate specificity for G-proteins) but which also produces a functional toxin A (Mehlig et al., 2001).

Clinical Illness

DISEASE *Clostridium difficile* is an excellent example of a classic opportunistic pathogen. In healthy persons, the intestinal flora is highly protective against infection by intestinal pathogens. However, when this protective flora is disrupted, most typically by antibiotics or other powerful drugs such as the anticancer agents, *C. difficile* is able to enter and colonize the colon. The organism begins to grow as the amount of antibiotic in the colon drops below the level that inhibits its growth. This occurs with toxigenic and nontoxigenic isolates, both of which form spores and are present in the environment. Patients that are colonized with *C. difficile* will have counts of >10^8 per gram in their feces. Although it has been suggested that the organism attaches to gut receptors, it is more likely that the organism grows throughout the lumen. As the organism grows in the colon, it begins to release toxin through autolysis as it reaches stationary phase. The toxins, particularly toxin A, likely target galactose-containing receptors once they are produced in the colon. Toxin A then is taken up through receptor-mediated endocytosis and generalized pinocytosis. Once in the cell, the toxic activity is expressed through the monoglucosylation of the GTP-binding protein Rho, resulting in a shutdown of the cytoskeletal system and cell rounding, and eventually cell death. In addition to its direct toxic action, toxin A likely is chemotactic, resulting in an intense inflammatory response (Triadafilopoulos et al., 1987; Triadafilopoulos et al., 1989; Pothoulakis et al., 1988). Other mechanisms may include the ability of toxin A to damage internal cellular organelles and activate other pathways that are independent of Rho inactivation (Liu et al., 2003). Inflammation also is triggered by the tissue damage caused by toxin A. The disruption of the mucosal membrane through direct toxic action and inflammation maintains the disruption of the microflora, resulting in further exacerbation of the disease.

The role of toxin B in the disease process is less understood than that of toxin A. Toxin B from typical A+/B+ isolates does not have any significant activity in the intestine of experimental animals. However, toxin B mixed with low nontoxic amounts of toxin A is lethal when given intragastrically to animals. In addition, toxin B is lethal when given to animals in which the mucosa has been damaged (Lyerly et al., 1985). These observations indicate a synergistic action between the toxins. Further, in immunodeficient mice containing transplanted human intestinal xenografts, toxin B causes intestinal permeability and severe inflammation (Savidge et al., 2003). In patients infected with atypical A–/B+ isolates, these observations suggest that toxin B may be capable of causing the tissue damage associated with the disease. However, the tissue damage may also result from the more extensive toxic properties of toxin B from A–/B+ isolates. This possibility is based on findings showing that when compared to toxin B from typical A+/B+ isolates, toxin B from A–/B+ isolates is 1) almost tenfold more lethal, 2) enterotoxic in animal models, and 3) targets a broader array of G-proteins (Rho, Rac and Ras for atypical toxin B versus just Rho for typical toxin B). In this regard, the atypical toxin B more closely resembles *C. sordellii* lethal toxin.

These differences may explain the ability of atypical isolates to cause disease in the absence of toxin A.

The clinical criteria for *C. difficile* disease has been defined as diarrhea in which no other cause has been established, loose or unformed stool that takes the shape of the container, three or more bowel movements per day, duration of at least 2 days, and a history of antibiotic or antineoplastic agents in the preceding 4–6 weeks (Gerding and Brazier, 1993). Patients do not typically develop abdominal pain, fever, ileus or constipation. The presence of toxin is strongly supportive of *C. difficile* disease. Metronidazole or vancomycin is typically used to treat the disease, and a positive response to these antibiotics often is considered confirmatory for *C. difficile* disease. However, these antibiotics are not specific for *C. difficile* and will act on other intestinal pathogens that may cause AAD.

Pseudomembranous colitis, the severe stage of the disease, is not observed as frequently as it was 20 years ago because of more accurate rapid diagnostic testing, greater awareness of the disease, and quicker implementation of therapy. The condition, however, still occurs and may develop following the onset of diarrhea, occasional fever and chills, and some bleeding. The pseudomembrane lesions are typically several millimeters in diameter and appear as small whitish yellow plaques along the mucosal wall. The presence of these plaques, which are composed of necrotic cells, debris, and leukocytes, is diagnostic for PMC. Inflammation probably plays a primary role in the onset of these lesions. In severe *C. difficile* disease, there is an intense inflammatory component, due in part to the tissue damage from the toxin activity and from the chemotactic properties of toxin A. *Clostridium difficile* disease is most commonly manifested as antibiotic-associated diarrhea. Of the fecal specimens submitted to the laboratory for *C. difficile* testing, 5–15% usually are positive, although a positivity rate of up to 25% may be observed during outbreaks. Although clinical isolates differ in the amount of toxin they produce in vitro, no correlation has been established between levels of in vitro toxin production and severity of disease.

Clostridium difficile disease most often is treated with oral metronidazole or vancomycin. In most instances, the therapy is effective, although relapse rates of up to 20% have been noted. Relapses generally occur within several weeks of remission, probably from residual spores that remain in the patient. On occasion, relapses can occur following infection with a different isolate. Relapses do not occur, as some have suggested, because of the development of resistant strains. Rather, they occur because the

normal flora has not become reestablished, and the colon still is vulnerable to infection. Relapses can occur multiple times, and different therapeutic approaches, including limited antibiotic therapy and probiotics have been used with some success.

Several studies have demonstrated the presence of *C. difficile* and its toxins in patients with ulcerative colitis or Crohn's disease. Therefore, patients with inflammatory bowel disease who do not respond to therapy should be screened for *C. difficile* toxin, especially if they have undergone recent antibiotic therapy (Greenfield et al., 1983; Keighley et al., 1982).

DIAGNOSIS The detection of pseudomembranes by endoscopic exam using procedures such as rigid proctoscopy, flexible sigmoidoscopy, or surgical exploration is considered diagnostic for *C. difficile* disease. Under these conditions, in vitro laboratory tests are used as supplemental tests to confirm the diagnosis. Endoscopic examination is not performed routinely in persons with antibiotic-associated diarrhea.

Bacterial culture, tissue culture assay, and detection of a nontoxic antigen or toxins A and B with antibody-based tests are used as in vitro diagnostic aids for *C. difficile* disease (Table 4). Bacterial culture is the most complicated of these tests, and it seldom is used because it is tedious, time-consuming, and inconsistent. The procedure requires expertise with anaerobic bacteriology, and the media must be high quality to ensure high recovery rates. Cefoxitin-cycloserine-fructose agar (CCFA) is the media of choice for this procedure, although some laboratories use selective broth media (e.g., brain heart infusion broth supplemented with cefoxitin and cycloserine). *Clostridium difficile* is not as strict an anaerobe as some other clostridia (e.g., *C. tetani*), but precautions (e.g., good technique, high quality media, fresh stool specimens, etc.) should be taken for higher recovery rates. Not all isolates of *C. difficile* sporulate to the same degree. However, in many instances, heat-shock or alcohol wash procedures can be used to improve recovery. *Clostridium difficile* gives flat rough colonies that grow to several millimeters in diameter, and colonies can be presumptively identified on CCFA. Other anaerobes, as well as nontoxigenic isolates of *C. difficile*, grow on CCFA. It has been our experience that roughly 25% of *C. difficile* isolates from patients with antibiotic-associated diarrhea are nontoxigenic. For this reason, presumptive colonies should be screened further for toxin production.

A number of *C. difficile* antigen tests are on the market. The antigen is glutamate dehydrogenase, a metabolic enzyme produced both by toxigenic and nontoxigenic isolates (Lyerly et al.,

Table 4. Methods and tests to detect *C. difficile* and its toxins.

Method	Entity detected	Advantages	Limitations	Available tests (and sources)
Bacterial culture	Organism	Most sensitive, specific	Efficiency varies from lab to lab, use of a stereomicroscope recommended	CCFA with horse blood preferred; 500μg of cycloserine and prereduction of medium increases the isolation rate of *C. difficile* (Remel, Lenexa, KS)
Latex agglutination	Glutamate dehydrogenase	Rapid, simple	Not extremely sensitive, does not distinguish between toxigenic and nontoxigenic strains of *C. difficile*	CDT (Becton Dickinson Microbiology Systems, Sparks, MD) Meritec *C. difficile* (Meridian Diagnostics Inc., Cincinnati, OH)
Membrane EIA	Glutamate dehydrogenase	Rapid, simple	More sensitive than latex agglutination, does not distinguish between tox+ and tox– strains	ImmunoCard *C. difficile* (Meridian Diagnostics, Inc., Cincinnati, OH) Triage *C. difficile* panel (Biosite Diagnostics, Inc., San Diego, CA)
Microwell EIA	Glutamate dehydrogenase	Rapid, simple, specific	More sensitive than latex agglutination, does not distinguish between tox+ and tox– strains	C. diff CHEK-30 (TechLab, Inc., Blacksburg, VA) C. diff CHEK-60 (TechLab, Inc., Blacksburg, VA)
Tissue culture	Toxin B	Sensitive, specific	Most assays require 24–48h to complete; toxin B can be inactivated, resulting in false-negative results	*C. difficile* Toxin/Antitoxin (TechLab, Inc., Blacksburg, VA) *C. difficile* Tox-B Test (Wampole Laboratories, Cranburry, NJ and TechLab Inc., Blacksburg, VA) *C. difficile* Toxititer (Bartels, Inc., Issaquah, WA) Cytoxi (Advanced Clinical Diagnostics, Toledo, OH)
Microwell EIA	Toxin A	Rapid, simple, specific	Some kits have higher sensitivity than others, some kits may yield indeterminant readings. Does not detect atypical A–/B+ isolates that cause disease	CD-Tox (Porton, Cambridge, England) Premier (Meridian Diagnostics, Inc., Cincinnati, OH) Prospect II C. difficile Toxin A microplate (Alexon-Trend, Sunnyvale, CA)*C. difficile* TOX-A TEST (Wampole Laboratories, Cranburry, NJ and TechLab, Inc., Blacksburg, VA) Toxin A EIA (Bartels, Inc., Issaquah, WA) Toxin CD Test (Becton Dickinson Microbiology Systems, Sparks, MD) VIDAS-CDA (bioMerieux Vitek, Hazelwood, MO) Triage C. difficile panel (Biosite Diagnostics, Inc., San Diego, CA)*C. difficile* Toxin A Microplate Assay (Remel, Lenexa, KS)
Membrane EIA	Toxin A	Rapid	Not as sensitive as microwell EIAs	*C. difficile* Toxin A (Oxoid, Inc., Ogdensburg, NY) Triage *C. difficile* Panel (Biosite Diagnostics, Inc., San Diego, CA)
OIA	Toxin A	Rapid, simple	Less sensitive than EIA	CdTOX A OIA (ThermoBiostar, Boulder, CO)
Microwell EIA	Toxins A and B	Rapid, simple, specific	Some kits have higher sensitivity than others, some kits may yield indeterminant readings	*C. difficile* TOX A/B II (Wampole Laboratories, Cranburry, NJ and TechLab, Inc., Blacksburg, VA) Cd Toxin A + B (Rohm Pharma, Darmstadt, Germany) Premier A + B (Meridian Diagnostics, Inc., Cincinnati, OH)

Abbreviations: CCFA, cycloserine-cefoxitin-fructose agar; EIA, enzyme immunoassay; and OIA, optical immunoassay.

1991). The newer membrane and microwell EIA antigen tests that are available are more sensitive than the latex formats that appeared on the market more than ten years ago (Lee et al., 2003). Because of their higher sensitivity, the newer tests have excellent negative predictive values and accurately rule specimens negative for *C. difficile*. For laboratories that perform tissue culture assays, these tests are valuable as screens to reduce the amount of diagnostic testing by tissue culture. Tissue culture assays are more tedious than the membrane and microwell EIAs and require 48 hours before ruling a specimen negative, compared to an hour or less for the antigen tests. For laboratories that perform EIA for toxin, the antigen tests can be used to identify patients who may have all the symptoms and conditions for *C. difficile* AAD, but who have only trace amounts of toxin owing to low expression or inactivation, and who are negative or borderline in toxin EIAs. The glutamate dehydrogenase (GDH) antigen is produced in much higher amounts than either toxin A or toxin B, and a positive test may alert the physician to monitor the patient and perform additional toxin testing if needed.

Diagnostic tests that detect toxin are highly suitable for confirming the presence of toxigenic isolates in a patient. Tissue culture assay and toxin-specific EIAs are available for this purpose. Tissue culture assays are considered to be the gold standard because of the high level of sensitivity. However, on occasion, fecal specimens cause cell rounding and cell stretching owing to factors other than toxins A and B (Lyerly et al., 1998). Therefore, tissue culture results should not be overinterpreted with specimens that cause cell stretching or rounding not neutralized by specific *C. difficile* antitoxin. The tissue culture assay has some disadvantages. It is tedious, results cannot be ruled negative for 48 h, and in-house methods often are not standardized. Cell lines that are commonly used include human foreskin, WI-38 lung fibroblasts, and CHO cells. A cutoff of 50–90% cell rounding is recommended in most tests, although some laboratories consider any cell rounding to be positive. Weakly positive specimens should be interpreted cautiously since freezing the specimen or extended storage conditions (>72 h) may lead to weak cytotoxic activity.

A large number of toxin-specific EIAs are available. Most detect toxin A, although several detect both toxin A and toxin B. Until recently, ELISAs that detect both toxin A and toxin B were believed to be unnecessary. Toxin A had been shown to be a very potent enterotoxin in experimental animal models, and it was an excellent diagnostic marker for toxigenic *C. difficile* in fecal specimens. In addition, there was some

question about the role of A–/B+ isolates in clinical disease. Until recently, only one A–/B+ isolate, CCUG 8864, had been described (Torres, 1991; Lyerly et al., 1992; Wilkins and Lyerly, 2003). Interestingly, though, toxin B from the 8864 isolate was more enterotoxic than toxin B from typical A+/B+ isolates. The appearance of true A–/B+ isolates from clinical specimens soon became apparent by testing specimens with a panel of tests that detected both toxins. These atypical isolates now have been identified in Japan, England, Canada, Israel, the Netherlands, France, and the United States. In Japan, most of the A–/B+ isolates have been associated with asymptomatic persons or only with mild diarrhea (Kato et al., 1998). In Canada, the United States and Europe, however, several deaths and outbreaks have been attributed to A–/B+ isolates (Lyerly et al., 1998; Alfa et al., 2000; Johnson et al., 2001; Kuijper et al., 2001; Pituch et al., 2001; Barbut et al., 2002; Limaye et al., 2002). In parts of Europe, approximately 5% of the clinical *C. difficile* isolates are A–/B+, and they occur in roughly 25% of the hospitals that have been surveyed. In general, the clinical significance of A–/B+ isolates can be summarized as follows: 1) A–/B+ isolates may cause disease that can be severe or fatal if not promptly diagnosed and treated; 2) the incidence of A–/B+ isolates appears to be low; 3) A–/B+ isolates are spread similarly to typical A+/B+ isolates, and 4) A–/B+ isolates do not react in toxin A-specific EIAs. They do react in EIAs that detect both toxins, are positive in tissue culture assays, and react in antigen tests. Atypical A–/B+ isolates do not react in toxin A-specific EIAs because they 1) carry a truncated *toxA* gene that lacks the portion of the toxin recognized by the antibodies used in the toxin A-specific ELISAs (Kato et al., 1999; Moncrief et al., 2000b), and 2) there is a stop codon located near the 5'-end of the truncated gene (Von Eichel-Streiber et al., 1999).

PCR for the amplification of the *toxA* and *toxB* genes has been proposed as an alternative to tissue culture and antibody-based assays for detecting toxigenic *C. difficile* in fecal specimens. In most of the studies, direct amplification using specific toxin gene primers resulted in a highly specific product that identified toxigenic *C. difficile* and did not detect *C. sordellii*, which produces crossreacting toxins (Kato et al., 1993; Gumerlock et al., 1993; Kuhl et al., 1993; Alonso et al., 1997; Karasawa et al., 1999). The correlation between tissue culture assay was >98%. There are PCR-inhibitory factors in stool specimens, and clean-up steps prior to amplification do improve performance. As an alternative, magnetic beads coated with monoclonal antibodies specific for *C. difficile*, but which crossreact with *C. sordellii* and *C. bifermentans*, have been used

as a capture and enrichment step, followed by PCR specific for *toxB* (Wolfhagen et al., 1994). In more recent studies, the reported sensitivity for PCR amplification of *toxB* when compared to tissue culture was 92% (Guilbault et al., 2002). When targeting *toxA* and *toxB* and using a real-time fluorescence-based multiplex PCR assay, the reported sensitivity was 97% (Belanger et al., 2002). For diagnostic purposes, if PCR testing is to be used, a positive result in the absence of any other test results must be interpreted cautiously. Many patients carry toxigenic isolates asymptomatically or they may have diarrhea due to causes other than *C. difficile*.

Treatment

As an initial step in the treatment of *C. difficile* disease, the causative antibiotic should be discontinued. In some instances, simply stopping the antibiotic has led to recoveries in 25–30% of patients with AAD. By stopping the offending antibiotic, the normal microflora begins to become reestablished, helping the intestine to recover. The most commonly used antibiotics for *C. difficile* are metronidazole and vancomycin, both requiring a 7–10 day course of therapy. Metronidazole is the preferred oral agent of therapy (Fekety, 1997; MacLaren et al., 1997; Reinke, 1998; Wilcox, 1998). Metronidazole, which is selectively toxic for anaerobic organisms and some microaerophiles, acts as an electron acceptor and causes biochemical lesions in the cell structure. In addition, metronidazole interferes with DNA synthesis in anaerobes. In the body, metronidazole is metabolized to the intermediate hydroxymetronidazole, which has a minimal inhibitory concentration (MIC) of 0.5–4.0 μg/ml, compared to 0.25–1.00 μg/ml for the parent compound (Bolton and Culshaw, 1986). Metronidazole is considerably cheaper than vancomycin, and it has been used more extensively for treating patients who have mild or moderate disease. Typically, oral doses of 250–500 mg four times daily is given.

Vancomycin, which inhibits cell wall synthesis by binding to precursors of the cell wall (perhaps D-alanylalanine), is highly bactericidal for *C. difficile*. This drug is not absorbed very well in the intestine, and high concentrations are achieved when it is administered orally. The typical dose range is 125–500 mg at 6-hour intervals, giving fecal levels in excess of 1 mg/ml, well above the MIC value for *C. difficile*. The higher dose should be reserved for patients who are critically ill (Fekety et al., 1989). Vancomycin is available in liquid and capsule form, both of which are highly effective. A primary concern is the overuse of vancomycin, leading to the development of vancomycin-resistant enterococci. For this reason,

and because of the high costs of vancomycin, metronidazole probably is used more often for *C. difficile* infections.

Other antibiotics shown to be efficacioius for treating *C. difficile* infections include bacitracin, teicoplanin, and fusidic acid, although these have not been used routinely for this purpose. Other approaches include the use of resins such as cholestyramine or colestipol that bind the toxins of *C. difficile*. The binding resins also have been used in combination with antibiotics to treat patients who relapse. Probiotics such as *Saccharomyces boulardii* and *Lactobacillus* have been used to treat patients with recurrent *C. difficile* disease. *Saccharomyces boulardii* is a nonpathogenic yeast that has been used for years in Europe as a probiotic for persons with intestinal illnesses. Some patients treated with *S. boulardii* show a decrease in the number of CFU and concomitant decrease in toxin levels, followed by clinical improvement within days to weeks. *Lactobacillus* GG, a human isolate, has been used in some patients with limited success. Fecal enemas, which are not used in the United States, and combinations of various intestinal bacteria, including *Streptococcus*, *Clostridium*, *Bacteroides* and nontoxigenic *C. difficile*, have been suggested as possible alternatives to the use of antibiotics as therapeutics. These studies are ongoing and results are mixed, but they present possible ways in which the normal flora is allowed to reestablish itself. In addition to antibiotics and probiotics, another therapeutic approach includes the oral administration of bovine gamma globulin against *C. difficile*. In studies performed by us and other researchers, hamsters were protected against challenge with toxigenic *C. difficile* when treated prophylactically with gamma globulin against the organism and its toxins. In human volunteers, results have shown that bovine gamma globulin administered orally retained its neutralizing activity against toxin A in the intestine (Warny et al., 1999). Therefore, it has been suggested that oral administration of gamma globulin against *C. difficile* may be particularly effective in high-risk populations such as those in Veterans Administration hospitals. For relapsing patients, combinations of no antibiotic therapy, an additional round of antibiotic therapy, and alternative treatments such as toxin-binding resins and probiotics (*Saccharomyces*, *Lactobacillus*, and fecal enemas) have been utilized (Bartlett, 1995).

An awareness of *C. difficile* disease and education of healthcare personnel about methods to reduce the incidence of infections represents an important step in reducing outbreaks of this disease. Measures that help reduce the incidence include 1) proper handwashing and the use of gloves, 2) disinfectants such as hypochlorite to

help reduce spores, 3) avoiding the overuse of antibiotics, and 4) accurate diagnosis and enteric isolation if possible (Barbut and Petit, 2001). Continued surveillance to identify increased incidence also helps to minimize possible outbreaks. Treatment of asymptomatic carriers is not recommended.

Important Points for Healthcare Professionals

For healthcare professionals, we believe the following points are especially relevant:

1) *Clostridium difficile* is the major cause of hospital-acquired diarrhea and colitis. 2) There is no indication that the incidence of *C. difficile* disease is decreasing. *Clostridium difficile* continues to cause outbreaks in hospitals and medical centers around the world. 3) This pathogen is spread from patient to patient by healthcare professionals. 4) The disease does not result from antibiotic-resistant isolates. *Clostridium difficile* is sensitive to the antibiotics that are associated with the disease and only begins to grow as the level of antibiotic in the intestine decreases. 5) Atypical A–/B+ isolates exist and cause disease in humans. For this reason, an ELISA that detects both toxins, or a tissue culture assay for toxin B, should be used as an in vitro diagnostic aid to confirm the presence of *C. difficile* toxin in fecal specimens. 6) Nontoxigenic isolates exist in hospitals and are spread in patient wards. For this reason, antigen tests should be confirmed with toxin tests. 7) Many patients become asymptomatic carriers of *C. difficile* after entering hospitals and being placed on antibiotic therapy. It is not understood why some persons become ill, whereas others remain asymptomatic. Identification and treatment of asymptomatic carriers is not recommended. 8) An awareness of *C. difficile* disease and education about methods to reduce the incidence represents an important step in reducing outbreaks of the disease.

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CHAPTER 1.2.23

Clostridium perfringens and Histotoxic Disease

JULIAN I. ROOD

Introduction

The clostridia are a diverse group of Gram-positive spore-forming bacteria that cause a variety of potentially fatal human diseases including gas gangrene, tetanus, botulism and pseudomembranous colitis. The common feature of all of these diseases is that spore formation is important in their epidemiology and that they are mediated by powerful protein toxins. Since these syndromes are best discussed on a systems-related basis, rather than by their causative bacterial species, the clostridial chapters have been divided into separate discussions on the neurotoxic (Neurotoxigenic Clostridia in this Volume), enterotoxic (The Enterotoxic Clostridia in this Volume) and histotoxic clostridia. For earlier reviews on these topics see Rood et al. (1997) and Fischetti et al. (2000). The reader is also referred to McLennan's classical review of histotoxic clostridial infections of man (MacLennan, 1962) and a more recent review of clostridial diseases of animals (Songer, 1996). The emphasis of this chapter, and those associated with it, will be on the pathogenesis of these clostridial diseases and the structure and function of the toxins. This chapter will focus on clostridial myonecrosis, or gas gangrene, caused by *Clostridium perfringens*. It will also include a discussion of the genetics and genomic analysis of this organism, which is the most intensively studied clostridial species.

Phylogeny

The clostridia are a very heterogenous group of bacteria that were originally classified in the same genus because they were Gram-positive rods that had the ability to form heat-resistant endospores and grew only under anaerobic conditions. The genus is now much more diverse and includes Gram-negatives, non-spore-formers and aerobes. Therefore, taxonomically the clostridia are much more disparate than most bacterial genera: if it wasn't for their medical significance, they would be broken up into many different genera. On the basis of their 16S rRNA sequences, they can be divided into 19 clusters that are intermingled with members of other genera (Collins et al., 1994; Stackebrandt et al., 1999). *C. perfringens*, along with *C. septicum* and *C. novyi,* as well as *C. botulinum* and *C. tetani*, belongs to cluster I, which also includes the type strain *C. butyricum* (Fig. 1). By contrast, *C. difficile* and *C. sordellii* belong to cluster XI. Even within species there is considerable taxonomic confusion. For example, 16S rDNA sequence analysis indicates that *C. novyi* types B and C and *C. histolyticum* probably represent a single species that is more closely related to *C. botulinum* types C and D than to *C. novyi* type A (Sasaki et al., 2001a). Subsequent phylogenetic analysis of the flagellin gene *fliC* indicates that *C. novyi* type B and *C. histolyticum* can be differentiated from one another (Sasaki et al., 2002).

It is time for the phylogenetic reclassification of the clostridia, but this process would present considerable difficulty for medical microbiologists, with *C. botulinum* types C, D and E having to be named as a different species. At the furthest extreme, if taxonomic rules were to be strictly enforced, the cluster I clostridia, including the type strain, would have to be moved to the genus *Sarcina*. Even if the genus *Clostridium* was to be retained and restricted to cluster I and II species, which is possible, then pathogens such as *C. difficile* and *C. sordellii* would still have to be given new genus names (Stackebrandt and Rainey, 1997). Clearly, the reclassification of the clostridia, although essential, is not going to be a simple task.

Genetics

The genetics of *C. perfringens* is highly developed, with conjugation and transformation methods well established. Several plasmids and mobile genetic elements have been identified and analyzed in considerable depth, and it is possible to genetically manipulate at least two
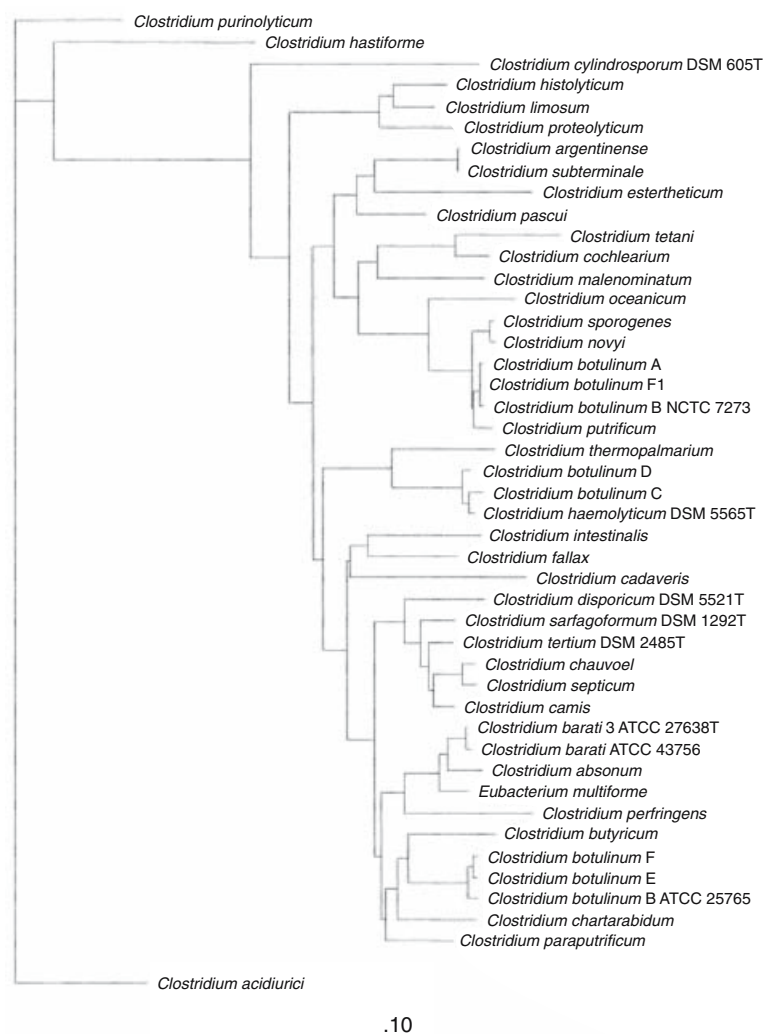
Fig. 1. Phylogenetic relationships of clostridial species within 16S rDNA cluster I. Pathogenic species are shown in bold. The bar represents 10% sequence divergence. From Stackebrandt et al. (1999), with permission.

strains of *C. perfringens* and to construct chromosomal mutants by homologous recombination (Lyras and Rood, 2000a).

Clostridium perfringens Plasmids

THE BACTERIOCIN PLASMID pIP404 AND CLONING VECTORS BASED ON pIP404. The 10,207-bp mobilizable plasmid pIP404 encodes 10 open reading frames (ORFs) and was the first *C. perfringens* plasmid to be completely sequenced (Garnier and Cole, 1988a). The *bcn* gene, which encodes the secreted bacteriocin BCN5, and the *uviAB* operon are both positively regulated at the transcriptional level by exposure to ultraviolet light (Garnier and Cole, 1988c). Although it was originally suggested that UviA may be involved in bacteriocin immunity, the UviA protein has sequence similarity to the transcriptional regulators BotR from *C. botulinum* (Marvaud et al., 1998a), TetR from *C. tetani*

(Marvaud et al., 1998b), and TxeR from *C. difficile* (Mani and Dupuy, 2001), which positively regulate botulinum toxin, tetanus toxin, and the *C. difficile* toxins A and B production, respectively. TxeR has been shown to be an alternative sigma factor that activates transcription of its target genes by binding to the RNA polymerase core enzyme and promoting its binding to a specific promoter target site (Mani and Dupuy, 2001; Mani et al., 2002). BotR and TetR are functionally interchangeable, and it is highly likely that the same would apply to TxeR and UviA. That is, these proteins are likely to form a functionally similar group of alternative sigma factors.

UviB is a potential cell membrane protein and may play a role in bacteriocin secretion (Garnier and Cole, 1988a). The *cop* gene encodes a putative membrane protein that is involved in copy number control (Garnier and Cole, 1988b). Similarly, plasmid replication is known to be dependent upon the basic protein product of the *rep*

gene (Garnier and Cole, 1988b). The minimal replication region of pIP404 comprises the *rep* gene and the region of repeated sequences that are immediately upstream. The plasmid also carries a *res* gene, which encodes a protein that belongs to the resolvase family of site-specific serine recombinases and is probably involved in the resolution of plasmid multimers (Garnier et al., 1987; Garnier and Cole, 1988a). Although the function of ORF10 is not known, there is a chromosomal variant located upstream of the regulatory *virRS* operon (Lyristis et al., 1994). Since these proteins have an N-terminal internal *N*-acetylmuramyl-L-alanine amidase domain (COG0860), ORF10 could be involved in the release of bacteriocin or other proteins from the cell. The role of the other three pIP404-encoded genes, ORFs 6, 7 and 9, is unknown.

Initial studies on the replication of pIP404 were carried out in *Bacillus subtilis* (Garnier and Cole, 1988b). However, with the advent of methods for the transformation of *C. perfringens* cells by electroporation (Allen and Blaschek, 1988; Scott and Rood, 1989), pIP404 was rapidly utilized for the construction of shuttle vectors (Sloan et al., 1992; Bannam and Rood, 1993). The most commonly used *Escherichia coli-C. perfringens* shuttle plasmids are those derived from pJIR418. This plasmid contains the ColE1-like replication region, multiple cloning sites, and *lacZ'* gene from pUC18, the *rep* gene and associated *C. perfringens* origin of replication from pIP404, and the *C. perfringens catP* and *erm*(B) genes, which encode resistance to chloramphenicol and erythromycin, respectively (Sloan et al., 1992). Modified shuttle plasmid derivatives of this plasmid include the deletion derivatives pJIR750, which encode chloramphenicol resistance, and pJIR751, which encodes erythromycin resistance (Bannam and Rood, 1993), the promoter probe vector pPSV (Matsushita et al., 1994), the expression vector pFF (Takamizawa et al., 2004), and the mobilizable shuttle vectors pJIR1456 and pJIR1457 (Lyras and Rood, 1998a). The latter two plasmids contain the *oriT* site from RP4, and as a result they can be used to introduce cloned genes into *C. perfringens* by conjugative mobilization from *E. coli*. Subsequent studies have shown that pJIR1457 can be introduced by conjugative mobilization from *E. coli* into both *C. botulinum* (Bradshaw et al., 1998), *C. difficile* (Mani et al., 2002) and other clostridial species (D. Lyras, M. Awad, and J. Rood, unpublished observation). Derivatives of pJIR418 have also been introduced into *C. paraputrificum* by electroporation (Sakka et al., 2003).

THE pCW3 FAMILY OF CONJUGATIVE TETRACYCLINE RESISTANCE PLASMIDS. Early studies on antibiotic resistance determinants showed that both tetracycline and chloramphenicol resistance genes could be carried on conjugative plasmids (Brefort et al., 1977; Rood et al., 1978). pCW3 is a 47-kb plasmid that carries an unusual tetracycline resistance determinant that consists of an operon containing two distinct tetracycline resistance genes, *tetA*(P) and *tetB*(P) (Sloan et al., 1994). The TetA(P) protein is a transmembrane efflux protein that confers tetracycline resistance by actively effluxing tetracycline from the cell. It is a distant member of the Major Facilitator Superfamily and has been extensively analyzed (Kennan et al., 1997; Bannam and Rood, 1999; Bannam et al., 2004). The TetB(P) protein is related to the Tet(M) protein from Tn*916* and confers tetracycline and minocycline resistance by a ribosomal modification mechanism (Sloan et al., 1994).

Expression of the tetracycline resistance encoded by the *tetA*(P)*tetB*(P) operon on pCW3 is induced by subinhibitory concentrations of tetracycline (Rood, 1983; Abraham and Rood, 1985a) via a complex process that is not well understood but is known to involve a host-encoded factor (Johanesen et al., 2001b). Induction involves transcriptional activation from a promoter located 529 bp upstream of the *tetA*(P) start codon, but no transcriptional activator has been identified (Johanesen et al., 2001a). Unusually, there is a transcriptional terminator located between the *tet*(P) promoter and the start codon, but the role that this terminator plays in the regulation process is not known. We have postulated that its function is to prevent overexpression of the TetA(P) transmembrane protein (Johanesen et al., 2001a).

All of the conjugative tetracycline resistance plasmids that have been analyzed from *C. perfringens*, irrespective of their geographic or host source, are closely related to pCW3 (Abraham and Rood, 1985b; Abraham et al., 1985c). Many of these plasmids are indistinguishable from pCW3 by restriction endonuclease analysis, others have small variations in restriction profile. These data provide good evidence that all conjugative R-plasmids from *C. perfringens* are derived from a single pCW3-like progenitor. Furthermore, the *tetA*(P) gene has been found in other clostridia, often in association with *tetB*(P), providing evidence that pCW3-like plasmids can be transferred to other clostridial species. Note that the *tetB*(P) gene has only been found in association with *tetA*(P) (Sasaki et al., 2001b; Lyras et al., 2004).

pCW3 has now been completely sequenced and shown to have little similarity to other known conjugative plasmids. The 47,263-bp plasmid has a G+C content of 27.6 mol% and encodes 52 putative genes, with only 35% of the predicted gene products having significant

similarity to proteins of known function (T. Bannam and J. Rood, unpublished observation). The region most likely to encode the conjugation-related functions is located in a 12-kb segment that is common to all of the known conjugative tetracycline resistance plasmids from *C. perfringens*. This region is also found in the conjugative enterotoxin plasmid from *C. perfringens* (Brynestad et al., 2001).

The plasmid pIP401 is a conjugative 53-kb plasmid that carries the genes for both tetracycline and chloramphenicol resistance (Brefort et al., 1977). Subsequent studies showed that pIP401 is effectively a derivative of pCW3 that carries the integrative mobilizable element Tn*4451*, which confers chloramphenicol resistance (Abraham and Rood, 1987). Other conjugative tetracycline and chloramphenicol resistance plasmids also consist of pCW3-like plasmids that carry Tn*4451*-like elements (Abraham et al., 1985c; Abraham and Rood, 1987).

THE TOXIN PLASMIDS OF *C. PERFRINGENS*. Traditionally, *C. perfringens* isolates have been divided into five toxin types (A to E), based on their ability to produce four major extracellular toxins (α, β, ε, and ι-toxins; Rood and Cole, 1991), although this classification system is outmoded owing to the identification of other, clinically significant, toxins such as the sporulation-associated enterotoxin, CPE (McClane et al., 2000) and β2-toxin (Gibert et al., 1997). Type A strains are the major human pathogens and, of the toxins used in the typing scheme, only produce α-toxin, which is chromosomally determined. Pulsed-field gel electrophoresis (PFGE) and genomic mapping of *C. perfringens* isolates of various toxin types led to the discovery that the β-, ε- and ι-toxin structural genes are plasmid determined (Canard et al., 1992; Cornillot et al., 1995; Katayama et al., 1996). Therefore, clearly, the toxin typing scheme in effect measures the acquisition of large toxin plasmids by type A strains of *C. perfringens* (Petit et al., 1999). Note that in veterinary and non-foodborne isolates the enterotoxin gene, *cpe*, is also found on a large plasmid, which can be conjugative (Brynestad et al., 2001). Little is known about the genetic organization of any of the large toxin plasmids from *C. perfringens*, although several sequencing projects are well under way.

The Tn*4451* Family of Integrative Mobilizable Elements

As already discussed, early studies led to the identification of pIP401, which carries a chloramphenicol resistance determinant. Conjugative transfer of pIP401 and selection for only tetracycline resistance led to the loss of the chloramphenicol resistance determinant at a high frequency, although some of the transconjugants retained the resistance gene (Brefort et al., 1977). Loss of resistance was associated with loss of a precise 6.2-kb region of DNA, which was subsequently designated as the transposable genetic element "Tn*4451*" (Abraham and Rood, 1987). All of the six chloramphenicol resistance plasmids that have been identified have been shown to carry similar elements (e.g., Tn*4452*), and closely related elements, designated as "Tn*4453a*" and "Tn*4453b*," have been identified on the chromosome of *C. difficile* (Lyras et al., 1998b).

Both Tn*4451* and Tn*4453a* have been sequenced and shown to comprise 6338-bp elements that encode six genes (Bannam et al., 1995; Lyras and Rood, 2000b; Fig. 2). One of these genes, *catP*, confers chloramphenicol resistance by encoding a chloramphenicol acetyltransferase. Although three of the remaining genes, *tnpV*, *tnpY* and *tnpW*, have no known function, *tnpX* is known to be required for excision and insertion of the element (Bannam et al., 1995) and that *tnpZ* encodes a mobilization, or Mob, protein (Crellin and Rood, 1997).

The members of the Tn*4451*/Tn*4453* family are not classical transposons (Adams et al., 2002). They should be more accurately referred to as integrative mobilizable elements (IMEs) because 1) they are excised and inserted by a site-specific recombination process that is encoded by the TnpX protein (Crellin and Rood, 1997; Lyras et al., 2004), which is a serine recombinase not a transposase, and 2) although they are not conjugative, they can be mobilized in the presence of a conjugative plasmid in a specific *oriT*-TnpZ-dependent manner (Crellin and Rood, 1998).

The TnpZ protein is a mobilization protein that has similarity to Mob/Pre mobilization proteins from plasmids from other Gram-positive bacteria (Crellin and Rood, 1998). An *oriT*, or RS$_A$ site is located immediately upstream of the *tnpZ* gene (Fig. 2). TnpZ acts as a DNA relaxase and nicks Tn*4451* at the *oriT* site, and in the presence of the appropriate exogenous conjugation machinery, a single strand of the replicon on which Tn*4451* is located is transferred to a suitable recipient cell. Mutation of either the *tnpZ* gene or the *oriT* site renders the mobilization

Fig. 2. Genetic organization of Tn*4451*. The six Tn*4451* genes are shown by the arrows, with the *oriT* or RSA site indicated by the circle. The directly repeated G$_A$ dinucleotides at the ends of the element are also shown.

system inactive, indicating that both components are required for conjugative mobilization. TnpZ also acts in trans, resulting in the mobilization of plasmids carrying the *oriT* site, even if those plasmids do not carry the *tnpZ* gene (Crellin and Rood, 1998). This mobilization process has been used to develop genetic tools for the analysis of the Tn*4451* insertion and excision process (Lyras et al., 2004).

The excision and insertion of Tn*4451* are catalyzed by TnpX, which is a member of the large resolvase family of site-specific serine recombinases (Smith and Thorpe, 2002). TnpX is an 82-kDa protein that has a functional N-terminal resolvase domain (Crellin and Rood, 1997). Most resolvases are ca. 20-kDa proteins that are involved in the resolution of either cointegrate intermediates of the replicative transposition process or plasmid multimers. They are site-specific recombinases that act via a serine residue located close to their N-termini, forming a phosphoserine intermediate and promoting DNA strand exchange at directly repeated target sites. Resolvases and closely related invertase enzymes cleave their targets in a directional manner, forming a 2-bp staggered cut at the core of the target site (Smith and Thorpe, 2002). The residues at the ends of Tn*4451* resemble the target sites of several invertases, and these residues are critical for the excision process (Crellin and Rood, 1997). TnpX-mediated excision of Tn*4451* from its target site results in the formation of a nonreplicating circular DNA molecule (Bannam et al., 1995) that is the circular intermediate of the transposition process (Lyras and Rood, 2000b). Formation of this intermediate brings together a 35 box located at the right end of the element and a 10 box at the left end of the element, forming a perfectly spaced *tnpX* promoter that we have postulated is required to ensure that the circular element is subsequently inserted onto a target site located on another replicon, such as a plasmid or the chromosome (Lyras and Rood, 2000b). In its inserted state this promoter is disrupted, and expression of the *tnpX* gene, and, therefore, excision, is dependent upon promoter sequences found outside the element, providing a mechanism for reducing the level of excision and preserving its more stable integrated state.

The N-terminal resolvase domain of TnpX is essential for its function; mutation of residues conserved in resolvase proteins, including the catalytic Ser-15 residue, eliminates the ability of TnpX to catalyze either the excision (Crellin and Rood, 1997) or insertion reactions (Lyras and Rood, 2000b). These reactions are distinct. Although the insertional target sites of Tn*4451* resemble the joint of the circular intermediate (Crellin and Rood, 1997), even containing central GA residues, TnpX binds much less efficiently to these target sites compared to the circular intermediate joint or the left or right ends of the element (Adams et al., 2004). These results imply that despite the fact that both excision and insertion involve the same resolvase-mediated catalytic activity, they are distinct processes, with different synaptic complexes being involved.

TnpX is the only transposon-encoded protein required for transposition of Tn*4451* (Lyras et al., 2004). Evidence for this conclusion came from in vivo studies that systematically involved the construction of deletions in each of the other Tn*4451* genes and from in vitro studies, which demonstrated that purified TnpX could catalyze excision in the absence of any other protein. Further studies have shown that TnpX binds to a region that encompasses the ends of the element and have led to the identification of the DNA binding region, between amino acids (aa) 492 and 597 (Adams et al., 2004). Detailed analysis of the TnpX protein and its derivatives has shown that it has at least two major DNA binding domains, the oppositely charged aa 533–583 region, which is essential for biological activity, and the cysteine-rich aa 597–707 C-terminal domain, which is not essential (Lucet et al., 2005).

Gene Manipulation in *C. perfringens*

There are two generally accepted protocols for the electroporation-mediated transformation of *C. perfringens* cells; one of which involves late logarithmic phase cells (Allen and Blaschek, 1990), the other involving lysostaphin-treatment of early logarithmic phase cells (Scott and Rood, 1989). Highest transformation frequencies are obtained with derivatives of strain 13, which lacks a restriction system. An alternative, especially for studies requiring an enterotoxin producing strain, involves the use of SM101 (a transformation-proficient derivative of NCTC 8798; Zhao and Melville, 1998). As already described, specific shuttle plasmids can also be introduced into *C. perfringens* by conjugation from *E. coli* (Lyras and Rood, 1998a).

Electroporation-mediated transformation of *C. perfringens* cells by suicide vectors has been used to generate chromosomal or plasmid mutants by homologous recombination. Double crossovers in genes such as the α-toxin structural gene *plc*, the perfringolysin O structural gene *pfoA*, and Tn*4451*-encoded *tnpZ* gene located on pIP401 have been constructed and used to analyze gene function (Awad et al., 1995; Bannam et al., 1995). Other studies have involved the construction of *colA* (Awad et al., 2000), *cpe* (Sarker et al., 1999) and *virR* (Shimizu et al.,

1994) mutants. Transposon mutagenesis with Tn*916* has also been used to isolate mutants in *C. perfringens*, but this method is complicated by the frequent occurrence of multiple insertions (Awad and Rood, 1997).

Genomics

C. perfringens is the only histotoxic clostridium to have been completely sequenced (Shimizu et al., 2002). The transformable virulent strain 13 comprises 3,031,430 bp, with a G+C content of 28.6 mol%, and potentially encodes 2660 proteins (Fig. 3). In addition, the sequence of the type strain ATCC13124 is complete but not yet published and that of the enterotoxin producer SM101 is nearing completion; both sequences are available from {The Institute for Genome Research website}. The strain 13 genome is a little smaller than that of most other *C. perfringens* isolates (Canard et al., 1992).
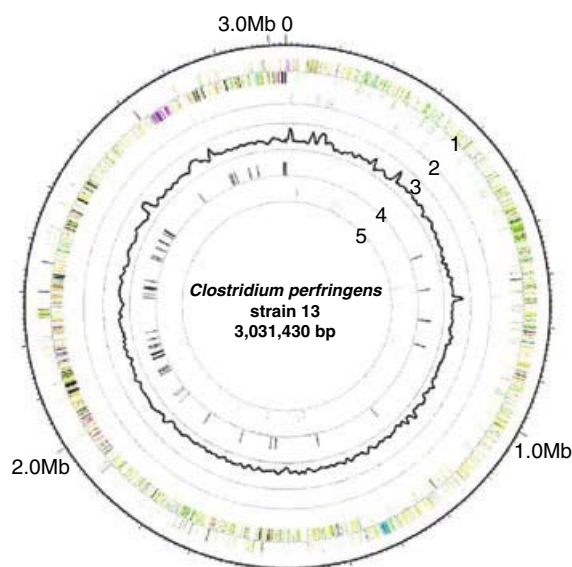
Fig. 3. Complete genetic map of the strain 13 chromosome. Ring 1 shows the coding sequence of each strand; ring 2 shows the positions of the 10 rRNA operons; ring 3 shows the variation in the mol% (G+C) content; ring 4 shows the position of the genes involved in sporulation and germination; and ring 5 shows the position of virulence-related genes. Legend to genes in ring 1: intermediary metabolism and fermentation (yellow), information pathways (pink), regulatory proteins (sky blue), conserved hypothetical proteins (orange), proteins of unknown function (light blue), insertion sequences and phage-related functions (blue), stable RNA molecules (purple), cell wall and cell processes (dark green), virulence genes (red), detoxification and adaptation (white), and sporulation and germination (black). From Shimizu et al. (2002), with permission.

Analysis of the strain 13 genome yielded considerable insight into the metabolism and pathogenic potential of *C. perfringens* (Shimizu et al., 2002). Consistent with the fact that *C. perfringens* ferments many different sugars and obtains all of its energy from substrate level phosphorylation, there were many homologues of genes encoding glycolytic enzymes but no homologues of genes encoding enzymes involved in the tricarboxylic acid cycle or proteins involved in the respiratory chain. The fact that *C. perfringens* must be provided with many different exogenous amino acids if it is to grow in minimal medium (Sebald and Costilow, 1975) was explained by the absence of genes required for the biosynthesis of most of these amino acids. Comparison with the *Bacillus subtilis* genome revealed the presence of 61 genes related to either sporulation or germination, although approximately 80 such genes were absent, especially those involved in spore coat biosynthesis and some germination genes. Many of the genes involved in the regulatory Spo0A phosphorelay were also missing. Clearly, much work remains to be done before the sporulation process in *C. perfringens* is understood; however, note that strain 13 does not sporulate as well as many other *C. perfringens* isolates.

At least 24 putative virulence genes were identified (Shimizu et al., 2002), but of these genes, only *plc* and *pfoA* have been shown to be involved in virulence (Awad et al., 1995; Awad et al., 2001). The designation of the remaining genes as virulence genes (Shimizu et al., 2002) must be regarded as highly speculative. For example, in addition to the *pfoA* gene, four genes were identified by comparative sequence analysis and proposed to encode hemolysins. However, two separate studies have shown that a *pfoA* mutant is nonhemolytic (Awad et al., 1995; Awad and Rood, 1997). Similarly, five separate hyaluronidase genes were identified and postulated to be involved in virulence (Shimizu et al., 2002) though only *nagH* has been shown to actually encode a functional hyaluronidase (Canard et al., 1994) and there is no evidence that hyaluronidase is involved in the pathogenesis of *C. perfringens* infections. Finally, numerous genes that encode the sensor histidine kinases and response regulators that comprise two-component signal transduction systems were identified (Shimizu et al., 2002). These genes include those encoding the well known VirS/R system (Rood, 1998).

A surprising finding of the strain 13 genome project was the discovery that this strain carries a previously unknown 54,310-bp plasmid, pCP13 (Shimizu et al., 2002). This plasmid carries many plasmid-related genes, a nonfunctional homologue of the *cbp2* β2-toxin gene (D. Fisher and

B. McClane, personal communication) but no homologues of known conjugation genes. Whether pCP13 is conjugative or confers any virulence properties on its host strain is not known.

Epidemiology

The critical epidemiological factor in histotoxic clostridial diseases is the production of endospores. These spores are resistant to environmental stress and can survive for long periods in the soil, retaining the ability to germinate and produce extracellular toxins when they are introduced into the nutrient rich environment of the human or animal body.

Spores are introduced into the body by contamination of gunshot or other wounds with soil and intestinal or fecal material. Gas gangrene is, therefore, the classical disease of human conflict or war. In the First World War and, prior to the introduction of penicillin, in the Second World War, gas gangrene was a major consequence of the contamination of gunshot wounds and a very significant cause of death (MacLennan, 1962). More modern causes of predisposing tissue damage include other traumatic injuries, such as those resulting from car accidents and intestinal surgery. Irrespective of the cause of the injury, the anaerobic environment created by the traumatic tissue damage provides the stimulus required to enable spore germination, vegetative growth, and the subsequent production of extracellular toxins. Notably, *C. perfringens* requires predisposing conditions to cause disease and is essentially a saprophytic soil organism. The primary purpose of the extracellular toxins and enzymes appears to be hydrolysis of organic material in the soil, presumably to provide nutrients for bacterial growth. The optimal temperature for the production of *C. perfringens* α-toxin, therefore, is 20°C not 37°C (Matsushita et al., 1996).

Clostridium perfringens-mediated Gas Gangrene

Gas gangrene can be defined as a histotoxic infection of muscle tissue that involves extensive toxemia, local edema, tissue necrosis, and gas production (MacLennan, 1962). The most common cause of gas gangrene is *C. perfringens*, although other clostridial species including *C. septicum*, *C. novyi*, *C. sordellii*, *C. histolyticum* and *C. fallax*, may be involved (MacLennan, 1962).

Infection of a superficial wound with a few vegetative cells or spores of *C. perfringens*, or another histotoxic clostridium, does not normally lead to histotoxic disease (MacLennan, 1962). In such circumstances the host tissues are highly oxygenated, which is not a suitable environment for the growth of these anaerobic bacteria. In addition, the ready supply of phagocytic cells provided by the normal inflammatory response helps to remove the infecting bacteria. However, when the wound is so traumatic that the blood supply to the tissues is affected, then the resultant ischemia provides ideal spore germination and growth conditions and limits the access of phagocytic cells to the invading anaerobes. If the inoculum is sufficiently large, as in a wound that results from intestinal perforation, then the bacteria may themselves help to create the appropriate anaerobic environment. However, the key factor in the development of disease is generally major disruption of the blood supply to the tissues (MacLennan, 1962).

Once *C. perfringens* cells are established and growing in the tissues they produce copious amounts of H_2 and CO_2 as a result of their fermentative metabolism, thereby helping to maintain an anaerobic environment. It is this bacterial gas production that gives the disease its common name of gas gangrene. In addition, at least two of the extracellular toxins produced by *C. perfringens*, α-toxin and perfringolysin O, act to prevent the influx of leukocytes from any blood vessels that remain intact, thereby protecting the developing focus of bacterial infection from the innate immune system. The absence of a significant inflammatory response is a hallmark of *C. perfringens*-mediated gas gangrene and has been recognized for many years (McNee and Dunn, 1917; MacLennan, 1962).

Extensive growth of the bacteria, followed by extracellular toxin production, leads to massive tissue necrosis. The onset of very severe and intense pain at the focus of infection is a very common feature and is a clear indication of major vascular collapse (MacLennan, 1962). Another key feature of this disease is tissues that are rapidly undergoing necrosis do not bleed (McNee and Dunn, 1917; MacLennan, 1962). Although the actual mechanisms by which these effects are mediated are not known, it has been postulated that they result from thrombosis of the microvasculature and resultant effects on the arterial blood supply (Bryant, 2003a). Disease progression is very rapid once a focus of infection is established, with previously healthy oxygenated tissue being quickly taken over by the advancing front of necrosis (McNee and Dunn, 1917; Bryant, 2003a). Death can occur within 24-48 h if the patient does not receive appropriate treatment. Death results from systemic toxemia, shock, and multiorgan failure, with extracellular toxins, primarily α-toxin, having effects on

systemic blood pressure and cardiac function and leading to hemodynamic collapse (Stevens, 2000; Stevens and Bryant, 2002).

Treatment involves a combination of massive doses of antibiotics, usually penicillin since *C. perfringens* is always susceptible to this antibiotic, coupled with surgical debridement of the wound. Amputation is often the only surgical option, as even treatment by antibiotics to which the bacterium is susceptible may fail to stop the progression of the disease, presumably because they do not get into the lesion. Rapid treatment and the location of the focus of infection are the key factors in patient survival (Stevens and Bryant, 2002).

Pathogenesis: Role of α-Toxin and Perfringolysin O

The demonstration in 1941 that *C. perfringens* α-toxin had lecithinase activity was the first time that a bacterial toxin had been shown to be an enzyme (MacFarlane and Knight, 1941). This toxin is the only toxin shown to be essential for *C. perfringens*-mediated gas gangrene (Awad et al., 1995). Although other toxins such as perfringolysin O and collagenase are not essential (Awad et al., 1995; Awad et al., 2000), these toxins, together with other extracellular toxins and enzymes such as hyaluronidase and sialidases, still may play an important role in the disease process.

Evidence for the essential role of α-toxin in disease comes from two independent studies. The construction and analysis of recombinant variants of α-toxin revealed that although the N-terminal domain (aa 1–249) could invoke an excellent antibody response in mice, these antibodies were not immunoprotective. By contrast, immunization with the C-terminal α-toxin domain (aa 247–370) could protect against experimental infection, providing evidence that

α-toxin is a critical toxin (Williamson and Titball, 1993). Recombinant vaccinia viruses producing the C-terminal domain could also be used to protect mice against a lethal challenge (Bennett et al., 1999). More recent studies have shown that immunization with the C-terminal α-toxin domain localizes the infection, reduces thrombosis and leukostasis, and prevents more systemic effects such as ischemia of the peripheral tissues (Stevens et al., 2004).

Definitive proof of the essential role of α-toxin came from genetic studies that involved the construction of an α-toxin null mutant by allelic exchange (Awad et al., 1995). The resultant mutant was avirulent in a mouse myonecrosis model, with virulence being restored when the mutant was complemented with a wildtype α-toxin gene. These studies and subsequent experiments also showed that α-toxin was the major toxin involved in the prevention of leukocyte influx into the infected lesion (Awad et al., 1995; Stevens et al., 1997; Ellemor et al., 1999; Fig. 4).

The precise mechanism by which leukocyte influx is inhibited is uncertain, but it appears to involve the α-toxin-mediated formation of intravascular aggregates of platelets and leukocytes (Bryant et al., 2000a). Injection of purified recombinant α-toxin into the muscle of rats leads to a rapid and specific occlusion of blood flow due to the formation of aggregates, initially in the venules but then in the arteries (Bryant et al., 2000a; Bryant et al., 2000b). Examination of these aggregates reveals that they stain strongly for the surface bound platelet adherence antigen P-selectin, suggesting that platelet aggregation is a major factor in their formation (Bryant et al., 2000a). This process involves the activation of a fibrinogen receptor, gpIIbIIIa or CD41, since monoclonal antibodies against gpIIbIIIa, or peptides that mimic fibrinogen, significantly reduce αtoxin-mediated platelet binding to granulocytes (Bryant et al., 2000b). By contrast, antibodies active against P-selectin have no effect on
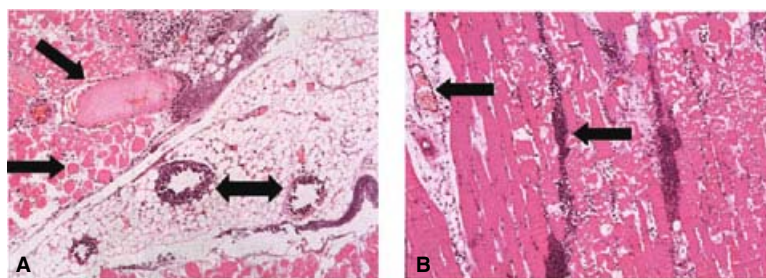
Fig. 4. Effect of *C. perfringens* α-toxin on muscle histopathology. Hematoxylin and eosin stained muscle sections obtained from mice infected with A (wild-type *C. perfringens*) and B) an isogenic plc mutant. The wild-type results in the extensive muscle necrosis and the presence of leukocyte aggregates and thrombosis in the blood vessels (arrows). None of these features is observed with the mutant; note the extensive leukocyte infiltration. From Ellemor et al. (1999), with permission.

α-toxin-induced aggregation. It has been suggested that agents that target gpIIbIIIa or its activation process might prove to be effective in the treatment of gas gangrene (Bryant et al., 2000b; Bryant et al., 2003b).

Further studies have shown that activation by α-toxin results in the depletion of internal calcium stores and calcium influx, which leads to activation of surface gpIIbIIIa, presumably by some sort of conformational change, and mobilization of stored gpIIbIIIa to the cell surface (Bryant et al., 2003b). Still unknown is how treatment of cells with α-toxin leads to these calcium-dependent changes, but the process does not appear to involve effects of the phospholipase C reaction product, diacylglycerol, on the protein kinase C cascade.

The α-toxin-mediated production of hydrolytic products such as diacylglycerol and ceramide has been implicated in the induction of proinflammatory changes in endothelial cells (Bunting et al., 1997). Treatment of human endothelial cell monolayers with purified α-toxin leads to the protein kinase C- and dose-dependent synthesis and release from the cells of the proinflammatory platelet activating factor (PAF) and the arachidonic acid derivative, prostacyclin, a strong vasodilator. These α-toxin treated cells have an increased capacity to bind to neutrophils, suggesting that the action of this toxin on endothelial cells may also play a role in leukocyte adhesion in the local vascular system and in vascular homeostasis. This process may be mediated by the upregulation of P-selectin expression in endothelial cells (Bunting et al., 1997). α-Toxin treatment also leads to the adhesion of rabbit neutrophils to fibrinogen and fibronectin (Ochi et al., 2002). Other studies have shown that α-toxin stimulates the upregulation of endothelial leukocyte adhesion molecule-1 (ELAM-1), intercellular adhesion molecule-1 (ICAM-1), and interleukin (IL)-8 expression, whereas perfringolysin O stimulates early ICAM-1 expression (Bryant and Stevens, 1996). For a comprehensive review of the effects of both α-toxin and perfringolysin O on thrombosis and leukostasis, see the excellent review by Bryant (2003a).

Genetic studies have also been carried out on the structural gene for the θ-toxin or perfringolysin O, *pfoA*, and the *colA* gene, which encodes κ-toxin or collagenase. Mutants that were unable to produce either perfringolysin O or collagenase still caused clostridial myonecrosis in mice, indicating that these toxins were not essential for virulence (Awad et al., 1995; Awad et al., 2000). However, perfringolysin O is still important in the disease process. Perfringolysin O upregulates the CD11b/CD18 adhesin on the surface of neutrophils (Bryant et al., 1993). Delayed spread of muscle necrosis was observed with the *pfoA* mutant as well as increased leukocyte infiltration, although not to the extent of the *plc* mutant (Ellemor et al., 1999). Finally, evidence that α-toxin and perfringolysin O have synergistic effects was obtained from virulence studies carried out by complementation of a *plc pfoA* double mutant (Awad et al., 2001). In these experiments complementation with the wild-type *plc* and *pfoA* genes produced a pathology that was more severe than that observed with just the *plc* gene.

Most strains of *C. perfringens* produce one or more sialidases, generally the smaller intracellular NanH enzyme (43 kDa) and the larger extracellular NanI enzyme (73 kDa) (Roggentin and Schauer, 1997; Takamizawa et al., 2004). The sequenced isolate of *C. perfringens*, strain 13, does not have *nanH* but has *nanI* and another potential extracellular sialidase gene, *nanJ* (Shimizu et al., 2002). Little is known about the role of any of these sialidases in disease, and no sialidase mutants have been constructed and virulence-tested. However, recent studies have shown that sialidase treatment may increase the sensitivity of cell cultures to α-toxin, suggesting that α-toxin and sialidase may also act synergistically (Flores-Díaz et al., 2004). It was proposed that surface gangliosides, which contain terminal sialic acid residues, protect the cell surface from the effects of α-toxin. These results are consistent with earlier studies that showed that Chinese hamster cell lines that had a mutation in UDP-glucose pyrophosphorylase and therefore were unable to produce UDP-glucose, a cell surface glycoconjugate precursor, were hypersensitive to the cytotoxic effects of α-toxin (Flores-Díaz et al., 1998).

Other workers have focused on the early stages of infection and have analyzed the action of α-toxin and perfringolysin O on macrophages (O'Brien and Melville, 2000; O'Brien and Melville, 2003; O'Brien and Melville, 2004). *Clostridium perfringens* cells have been shown to survive in a particular murine macrophage cell line, J774-33, where they escape from the phagolysosome and enter the cytoplasm (O'Brien and Melville, 2000). Phagocytosis of *C. perfringens* cells appears to involve receptors that may interact with mannose or glucuronic acid residues present in the *C. perfringens* capsule (O'Brien and Melville, 2003). Subsequent studies carried out on mouse peritoneal macrophages with *pfoA* and *plc* mutants suggest that both α-toxin and perfringolysin O are important for the survival of *C. perfringens* inside these cells, with perfringolysin O, and to a lesser extent α-toxin, also being important in escape from these macrophages (O'Brien and Melville, 2004). However, these data, and the subsequent mouse

virulence studies, need to be confirmed by complementation with the wildtype toxin genes. It was suggested that escape from the macrophage was important in the early stages of infection when cell numbers were lower. Evidence to support this hypothesis was obtained from *in vivo* studies carried out in mice using a relatively low infective dose. Under these conditions, both the *pfoA* and *plc* mutants had a lower survival rate in the muscle tissue(O'Brien and Melville, 2004).

Toxin Structure-Function Relationships

α-Toxin

The mature *C. perfringens* α-toxin is a 370-aa zinc metallophospholipase C. Zinc ions are required for enzymic activity, and calcium ions are essential for binding to the lipid membrane (Moreau et al., 1988). The toxin has phospholipase C activity, hydrolyzing phosphatidylcholine to diacylglycerol and phosphorylcholine and also has sphingomyelinase activity, hydrolyzing sphingomyelin to produce ceramide (Titball and Rood, 2000; Ochi et al., 2004). Both catalytic activities are the reflection of the same active site, which is located in the N-terminal domain of the toxin. The end products are important cell signaling molecules. Diacylglycerol is capable of activating the arachidonic acid pathway and the protein kinase C regulatory cascade (Titball and Rood, 2000), and ceramide causes apoptosis (Ohanian and Ohanian, 2001). The hemolytic activity of α-toxin is related to the presence of a distinct C-terminal domain and sphingomyelinase activity, which leads to α-toxin-induced activation of the sphingomyelin metabolic cascade (Ochi et al., 2004).

Determination of the crystal structure of α-toxin has revealed considerable information about its mode of action. The toxin consists of two major domains (Fig. 5), an N-terminal domain (aa 1–246) that is α-helical and contains the phospholipase C active site and a C-terminal domain (aa 256–370) that primarily consists of β-sheets (Naylor et al., 1998). These domains are joined by a short flexible linker (aa 247–255). The N-terminal domain consists of nine α-helices that are folded in a very similar manner to the nontoxic *Bacillus cereus* phosphatidylcholine-preferring phospholipase C (PC-PLC), which does not have the C-terminal domain. This domain contains an active site that binds three zinc ions.

The immunoprotective C-terminal domain is essential for toxicity and hemolytic activity, consists of two four-stranded β-sheets, and contains a key calcium-binding site (Fig. 5). This domain
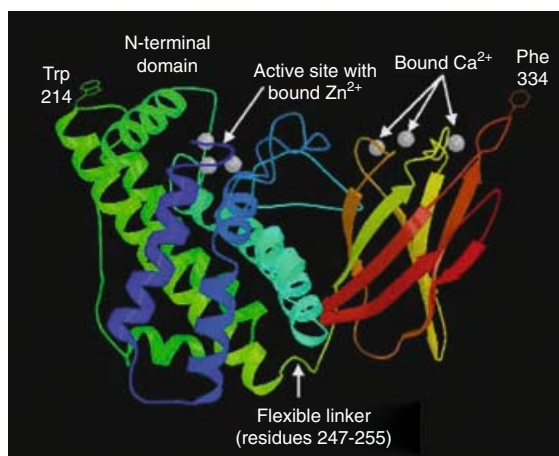
Fig. 5. Crystal structure of *C. perfringens* α-toxin. The crystal structure is represented as a ribbon diagram, with a color transition from the N-terminus (blue) to the C-terminus (red). As shown, the active site is in the α-helical N-terminal domain and the calcium-binding site is in the β-sheet C-terminal domain. From Fig. 2 of Titball and Rood (2000), with permission.

is a structural homologue of eukaryotic C2-lipid binding domains such as the C-terminal domain of human pancreatic lipase and the N-terminal domain of soybean lipoxygenase-1, which have an eight-stranded β-sandwich structure (Guillouard et al., 1997; Naylor et al., 1998). C2-domains are present in many eukaryotic signaling proteins and are involved in lipid-second messenger signal transduction pathways. The α-toxin C2 domain is required for the binding of the toxin to lipids at the host cell membrane surface and may mimic host cell proteins with similar C2 domains.

Further evidence that the C-terminal domain conferred hemolytic activity and cellular toxicity was obtained from domain swapping experiments between the *C. perfringens* α-toxin and the phospholipase C from *Clostridium bifermentans* (Jepson et al., 1999). The latter enzyme has a similarly sized C-terminal domain and equivalent phospholipase C activity on egg yolk emulsion but is 100 times less toxic. A hybrid protein comprised of the N-terminal domain of the *C. bifermentans* enzyme and the C-terminal domain of the *C. perfringens* enzyme ($N_{bi}C_{\alpha}$) was shown to be significantly more hemolytic and toxic for mice than the former enzyme. When similar domain swapping experiments were carried out between *C. perfringens* α-toxin and phospholipase C from *Bacillus cereus*, which does not have a C-terminal domain, the resultant $N_{bc}C_{\alpha}$ fusion protein was also hemolytic, although at only 0.1% of the wildtype α-toxin level (Nagahama et al., 2002). Furthermore, when separate nonhemolytic polypeptides consisting of the

N-terminal ($C_{\alpha1-250}$) and C-terminal ($C_{\alpha251-370}$) α-toxin domains were incubated together, hemolytic activity was restored. Similar complementation results were obtained by other workers using α-toxin mutants (Guillouard et al., 1997). These results are consistent with the observation that the C-terminal domain is required for α-toxin to bind to erythrocyte membranes.

Further comparative analysis of the wild-type *C. perfringens* and *C. bifermentans* enzymes revealed that in the C-terminal domain of the α-toxin there were three aa that appeared to be involved in membrane recognition but were different in the *C. bifermentans* enzyme (Jepson et al., 2001). Conversion of the α-toxin to a derivative that had the *C. bifermentans*-derived sequences (D269Y, Y331L and F334I) demonstrated that Tyr-331 and Phe-334 were both essential for full cytotoxic activity. The D269Y substitution altered the ability of the protein to bind Ca^{+2}; it had minimal effect on phospholipase C activity but led to increased cytotoxic activity, presumably because of an enhancement of the ability of the toxin to bind to the cell membrane (Jepson et al., 2001). These residues are all located on the putative membrane-binding surface of the α-toxin C-terminal domain.

Clostridium perfringens α-toxin appears to have both active and inactive conformations, with calcium binding being required for activation. There are three low affinity Ca^{+2} binding sites in the C-terminal domain, Ca1, Ca2 and Ca3 (Naylor et al., 1999). Asp-269 and Asp-336 have been shown to be required for the Ca^{+2} activation process (Guillouard et al., 1997) and are involved in Ca^{+2} binding at Ca1, along with Gly-271 and Ala-337 (Naylor et al., 1998). The Ca2 site involves Asp-293, Asn-294, Gly-296 and Asp-298, whereas Thr-272, Asp-273, Asn-297 and Asp-298 are implicated at Ca3. Note that amino acids that are adjacent in the linear amino acid sequence are involved in Ca binding but at different sites (Naylor et al., 1999). These authors suggested that if these sites are involved in binding to both Ca^{+2} and the host membrane, then the active site will be able to bind to its phosphatidylcholine substrate without disrupting the phospholipid membrane. Other experiments have shown that mutation of Asp-269, Asp-336, Tyr-275 and Tyr-307, and other residues in the C-terminal domain, significantly affects sphingomyelinase activity and toxicity (Alape-Girón et al., 2000).

In addition, there are two important surface-exposed hydrophobic residues, Trp-214, in the N-terminal domain, and Phe-334, in the C-terminal domain. When the toxin-membrane interface is modeled (Naylor et al., 1998), these residues appear to be inserted into the phospholipid bilayer, implying that they are involved in interactions with the cell membrane. PC-PLC, which is not hemolytic or toxic, does not have either the C2 domain or Trp-214.

Random mutagenesis has also been used to identify residues in the C-terminal domain that are required for biological activity. The three α-toxin mutants (D293S, K330E and D305G) analyzed had reduced hemolytic and cytotoxic activity (Walker et al., 2000). The D293S enzyme was Ca^{+2}-activated, which is consistent with this mutated protein having a reduced ability to bind Ca^{+2} and is in agreement with previous conclusions that Asp-293 was involved in Ca^{+2} binding at Ca2 (Naylor et al., 1999).

α-Toxin exists in two conformational forms, known as the open and closed conformations (Naylor et al., 1999). The original crystal structure was from an enzyme with the active site open (Naylor et al., 1998). Subsequently, the structure of an enzyme in the closed inactive conformation was determined (Eaton et al., 2002). Analysis of this structure provided evidence that movement of two loops in the N-terminal domain is critical for the opening and closing of the active site of the enzyme. It was postulated (Eaton et al., 2002) that the previously characterized T74I (Nagahama and Sakurai, 1996) and F69C (Guillouard et al., 1996) α-toxin mutants had reduced activity because these amino acid substitutions altered the conformation of loop 1 in the N-terminal domain.

The *C. absonum* α-toxin has similar phospholipase C activity to the *C. perfringens* α-toxin and is 1.5 times more hemolytic but only has half the toxicity (Clark et al., 2003). Comparative analysis of the crystal structures revealed a similar overall structure to the open form of *C. perfringens* α-toxin. A major difference was observed in the conformation of the aa 72–93 loop, which is more prominent in the *C. absonum* toxin; this change suggested a role for W84 in membrane binding. Conserved residues previously shown (Nagahama et al., 1995; Nagahama et al., 1997; Nagahama et al., 2000; Guillouard et al., 1996; Guillouard et al., 1997) to be involved in Zn coordination in the *C. perfringens* α-toxin (Trp-1, His-11, His-68, His-126, His-136, His-148 and Glu-152) and to be essential for enzymic activity are conserved in the *C. absonum* toxin (Clark et al., 2003). Note that the results of extensive site-directed mutagenesis studies on the *C. perfringens* α-toxin are summarized in two major reviews (Titball and Rood, 2000; Titball and Rood, 2002).

A divergent *C. perfringens* α-toxin has been characterized from a strain (SWCP) of *C. perfringens* isolated from a swan that died from a gastrointestinal *C. perfringens* infection (Justin et al., 2002). This variant toxin had 57–58 aa sequence changes compared to other *C. perfringens* α-toxins, with the altered aa distributed

relatively evenly throughout the protein. The active site was conserved. By comparison, other α-toxins from avian strains of *C. perfringens* had only one to six sequence changes (Sheedy et al., 2004). The SWCP α-toxin had an altered substrate specificity but was still hemolytic. Examination of its crystal structure revealed that it was similar to that of the open form of the previously determined α-toxin structure (Justin et al., 2002). It had the usual three zinc ions at the active site as well as the β-sandwich C-terminal domain. However, there were sequence and structural changes in one of the three Ca^{+2} binding pockets and its associated loop.

Perfringolysin O

Perfringolysin O is member of the cholesterol-dependent cytolysin (CDC) family of pore-forming cytotoxins, which was previously known as the "thiol-activated cytolysin family." The family includes perfringolysin O, streptolysin O, listeriolysin and pneumolysin (Billington et al., 2000). These toxins are closely related 40–60 kDa polypeptides that bind to cholesterol residues on the target cell membrane. Perfringolysin O has been shown to bind specifically to lipid rafts, which are cholesterol-rich domains in mammalian cells (Waheed et al., 2001; Ohsaki et al., 2004). Like other members of this family, perfringolysin O has an essential tryptophan-rich undecapeptide sequence, ECTGLAWEWWR, located in its C-terminal region (aa 430–440). Replacement of these tryptophan residues with phenylalanine, but not replacement of tryptophan residues located outside the undecapeptide, significantly reduced the ability of the resultant toxin to bind to and lyse red blood cells (Sekino-Suzuki et al., 1996), although subsequent studies have shown that these tryptophan residues are not required for cholesterol binding (Shimada et al., 1999). Modification of Cys-431 with a bulky thiol-blocking reagent also reduces the ability of the toxin to bind to membranes (Iwamoto et al., 1990). However, studies on other CDCs showed that cysteine to alanine replacements had little effect on biological activity (Michel et al., 1990). Therefore, the conserved thiol group of CDCs is not required for biological activity, which is why they are no longer known as thiol-activated cytolysins. Analysis of the structure of perfringolysin O explains these results as it predicts that bulky chemical modification of Cys-459 would lead to conformational changes in the Trp-rich loop and prevent cholesterol binding (Rossjohn et al., 1997). Chemical modification of the histidine residues in perfringolysin O also reduces activity and oligomerization in the cell membrane (Nakamura et al., 1999). Further studies have shown that the last

21 aa of perfringolysin O (aa 452–472) are required to maintain the native conformation of perfringolysin O, and deletion of the last three aa significantly reduces cholesterol binding and hemolysis (Shimada et al., 1999).

Production of perfringolysin O occurs in early logarithmic growth phase and is dependent upon the VirS/VirR two-component signal transduction system (Rood, 1998). The toxin is synthesized with a leader peptide sequence, which is cleaved upon passage through the cell membrane. The mature 52.7-kDa (472 aa) toxin has the ability to lyse red blood cells from many species and is the reason for the clear zone of hemolysis observed around *C. perfringens* colonies on blood agar. At the target cell surface the toxin forms aggregates that penetrate the phospholipid bilayer, form an oligomeric pore, and eventually lead to cell lysis.

The crystal structure of the toxin has been determined (Rossjohn et al., 1997) and the protein shown to have four distinct domains (Fig. 6), with the undecapeptide being located at the tip of domain 4. The toxin has a highly elongated structure that is rich in β-sheets (40%). The peripheral location of the undecapeptide prompted the suggestion that the tip of this domain was responsible for membrane insertion and oligomerization to form the pore that traversed the membrane (Rossjohn et al., 1997). Subsequently, fluorescence spectroscopy and cysteine scanning mutagenesis studies were used to identify regions of perfringolysin O that are no longer sensitive to water when the toxin binds to the cell membrane (Shepard et al., 1998; Shatursky et al., 1999). These regions are located in domain 3 of the toxin, which consists of a mixture of α-helices and β-sheets. Similar analysis has shown that domain 4 does not span the target membrane, only the hydrophobic tips of this domain make contact with the phospholipid bilayer of the target cell (Ramachandran et al., 2002; Czajkowsky et al., 2004). Furthermore, domain 4 does not make significant contact with adjacent monomers in the membrane-inserted oligomer (Ramachandran et al., 2002). This domain is still critical for cholesterol binding, although the precise nature of that interaction remains to be determined.

The current model for the insertion of perfringolysin O into the cell membrane involves the transition of α-helices in domain 3 of the soluble monomer to form two extended amphipathic antiparallel β-sheets in the membrane-inserted oligomeric complex (Fig. 6). The end-result is a membrane-spanning dual β-hairpin structure (Shatursky et al., 1999). The major theoretical problem with this model is that even after binding of the tips of domain 4 to the membrane, the amphipathic β-hairpins in domain 3 are too far
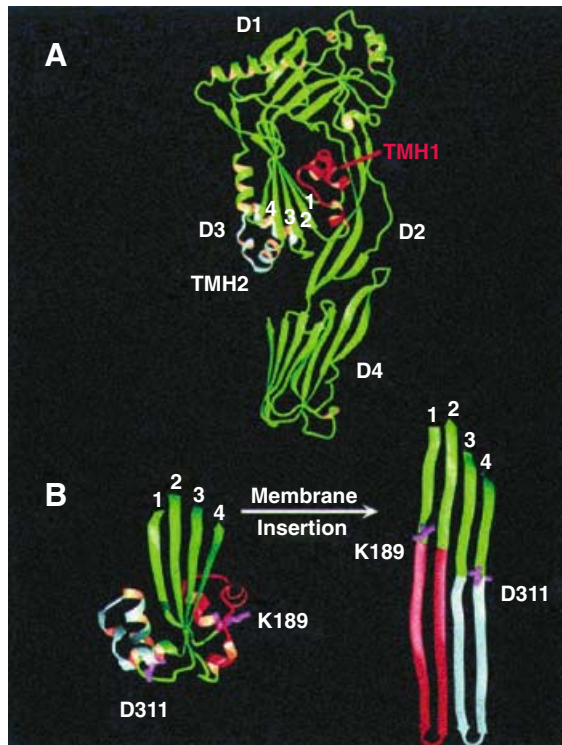
Fig. 6. Crystal structure of perfringolysin O and conformational transitions in the insertion of perfringolysin O into the cell membrane. A) A ribbon representation of perfringolysin O is shown, with the domains indicated. The conserved undecapeptide is located at the extended tip of domain 4. B) Conformational change of transmembrane helices 2 and 3 in domain 3 leads to the formation of a dual extended β-hairpin structure. From Fig. 4 of Shatursky et al. (1999), with permission.

Fig. 7. Schematic model of the prepore to pore transition of perfringolysin O. It is proposed that the vertical collapse of domain 2 brings the transmembrane domains of domain 3 close enough to the cell surface to enable membrane penetration by the amphipathic β-helices. Note that of the six residues tested, Q433 is the only residue quenched by the formation of the prepore complex. From Fig. 8 of Czajkowsky et al. (2004), with permission.

from the surface of the membrane to undergo membrane insertion. However, recent studies (Czajkowsky et al., 2004) involving time-lapse atomic force microscopy have shown that upon binding to the membrane, conformational changes in domain 4 lead to a novel vertical collapse of the elongated perfringolysin O structure (from 113 Å to 73 Å), bringing the domain 3 β-hairpins close enough to traverse the membrane (Fig. 7). Collisional quenching studies provide further evidence for this conclusion by demonstrating that after it makes contact with the cell membrane, the toxin monomer remains upright and does not bring domain 3 closer to the surface by a tilting-like mechanism. It is postulated that contraction of the extended structure of domain 2 is responsible for the vertical contraction that leads to membrane insertion (Czajkowsky et al., 2004).

Finally, a prepore complex appears to be formed prior to the insertion of the β-hairpins into the membrane (Shepard et al., 2000). Recent studies suggest that perfringolysin O is primarily a dimer in solution (Solovyova et al., 2004). The final pore is proposed to result from hydrogen-bonding between adjacent β-hairpins in the oligomeric membrane-bound complex, forming a β-barrel structure that contains about 50 monomers (Shepard et al., 1998; Shatursky et al., 1999). The oligomerization process appears to result from the formation of a monomer-monomer interface formed by a previously buried β-strand (from domain 3) in each monomer (Ramachandran et al., 2004). The formation of this interface involves a φ-stacking interaction between the aromatic rings of Tyr-181 and Phe-318, of the β1 and β4 strands, respectively, of domain 3. The trigger for the conformational changes involved in this process is contact of domain 4 with the cell membrane.

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CHAPTER 1.2.24

The Genera *Desulfitobacterium* and *Desulfosporosinus*: Taxonomy

STEFAN SPRING AND FRANK ROSENZWEIG

Introduction

Members of the genera *Desulfitobacterium* and *Desulfosporosinus* represent a clade of strictly anaerobic, rod-shaped, and sporeforming bacteria within the phylum Firmicutes, previously also known as the "low G+C Gram-positive bacteria."

The taxonomic and environmental significance of microorganisms belonging to this group escaped the attention of microbiologists for a long time and was only recently revealed, although the first representative species was already isolated in 1959. This species, however, was wrongly described as *Desulfovibrio orientis* (Adams and Postgate, 1959). Later the taxonomic affiliation of this bacterium to the Gram-positive sulfate reducers was recognized and it was reclassified as *Desulfotomaculum orientis* (Campbell and Postgate, 1965). Under this name it was included in the Approved Lists of Bacterial Names (Skerman et al., 1980). In the course of a phylogenetic analysis of the genus *Desulfotomaculum*, the separate taxonomic position of this species was revealed and the novel genus *Desulfosporosinus* was proposed with *Dsp. orientis* as type species (Stackebrandt et al., 1997). Some years earlier, Utkin et al. (1994) had already established the genus *Desulfitobacterium* with the type species *Dba. dehalogenans*. The special feature of this species was the reductive dechlorination of halogenated hydrocarbons. At that time this metabolic capability was only known in very few strict anaerobic bacteria. Since then, substantial progress was made in the isolation and description of novel strains representing the genus *Desulfitobacterium*, mainly caused by the growing interest in microorganisms being able to degrade and detoxify halogenated environmental pollutants under anoxic conditions. Currently, five different names of *Desulfitobacterium* species are validly published.

Besides halogenated compounds, several members of this group can also utilize heavy metals, in particular arsenic, as electron acceptor. Within this clade, strain Orex-4 was the first representative for which arsenate reduction was demonstrated. It was originally described as *Desulfotomaculum auripigmentum* (Newman et al., 1997) but has been recently reclassified as *Desulfosporosinus auripigmenti* (Stackebrandt et al., 2003). The capacity to reduce arsenic (As[V]) was later also reported in several strains belonging to the genus *Desulfitobacterium* (Niggemeyer et al., 2001).

Because of their unique metabolic potential, the environmental significance of species belonging to the genera *Desulfitobacterium* and *Desulfosporosinus* is obvious and has been demonstrated in numerous studies (e.g., Robertson et al., 2000; Breitenstein et al., 2001; Lanthier et al., 2001; Nevin et al., 2003). In the future, members of both genera could play a major role in the bioremediation of contaminated sites by the degradation of anthropogenic halogenated compounds or the removal of toxic heavy metals.

Common Traits and Differentiation

According to a recently established taxonomic framework based on the comparative analysis of 16S rRNA gene sequences, the genera *Desulfitobacterium* and *Desulfosporosinus* are affiliated to the family Peptococcaceae within the order Clostridiales (e.g., Boone et al., 2001). The main phenotypic traits characterizing both genera are in good agreement with this phylogenetic placement and include a strict anaerobic metabolism, a Gram-positive cell wall structure characterized by a murein sacculus, motility by means of flagella, and the ability to form endospores. Despite their Gram-positive cell wall architecture many species of this group stain Gram-negative or Gram-variable owing to an unusual thin peptidoglycan layer.

Phylogeny and Related Genera

Members of the genera *Desulfitobacterium* and *Desulfosporosinus* form a coherent phylogenetic group within the Peptococcaceae independent of the algorithm used for tree reconstruction (Fig. 1). The similarity values among 16S rRNA
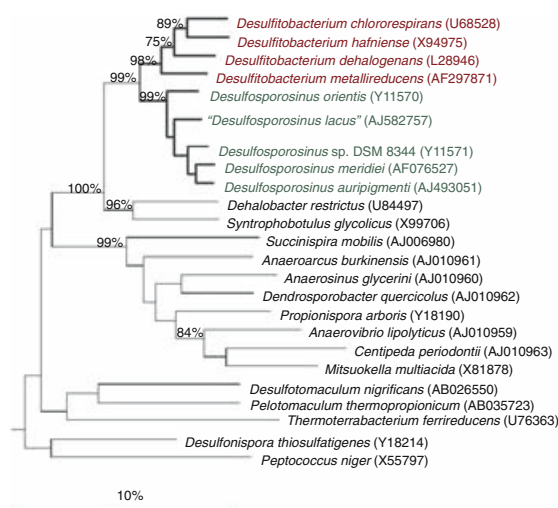
Fig. 1. Phylogenetic tree based on almost complete 16S rRNA gene sequences showing the positions of members of the genera *Desulfitobacterium* and *Desulfosporosinus* among type species of the family Peptococcaceae. The GenBank/EMBL accession number for each sequence is shown in parenthesis. The tree was reconstructed using the ARB program package (http://www.arb-home.de). It is derived from a distance matrix reconstructed by using the neighbor joining method of Saitou and Nei (1987). Phylogenetic distances were calculated as described by Jukes and Cantor (1969). The sequences of *Bacillus subtilis* (accession no. [{www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide&cmd=search&term=AJ27635}{AJ27635}]) and *Clostridium butyricum* (accession no. [{www.ncbi.nlm.nih. gov/entrez/query.fcgi?db=nucleotide&cmd=search&term= M59085}{M59085}]) were used to define the root (not shown). Only bootstrap values above 80% (1000 resamplings for each node) are shown at the respective branching points. Scale bar represents 10% estimated sequence divergence.

gene sequences within this cluster are 92.9–99.7%. The monophyletic origin of both genera is supported by the phylogenetic comparison of dissimilatory sulfite reductase genes (*dsr*AB). The DsrAB sequences of *Desulfitobacterium hafniense*, *Desulfitobacterium dehalogenans* and *Desulfosporosinus orientis* form a common branch and cluster together with several *Desulfotomaculum* species (Klein et al., 2001). Hence, it can be assumed that representatives of the genus *Desulfitobacterium*, although unable to reduce sulfate, evolved together with *Desulfosporosinus* species from a common sulfate-reducing ancestor. Despite this close phylogenetic relationship, members of both genera do not intermix but form well separated, distinct lineages supported by high bootstrap values. The clear-cut phylogenetic delineation between both genera is also illustrated by a set of signature nucleotides specific for the 16S rRNA genes of each particular genus (Stackebrandt et al., 2003).

The bootstrap values shown in the dendrogram tree clearly illustrate that a stable phylogenetic

relationship exists only with the genera *Dehalobacter* and *Syntrophobotulus*. Type species of other genera within the family Peptococcaceae are only distantly related to *Desulfitobacterium* or *Desulfosporosinus* species. On the other hand, certain members of the genus *Desulfotomaculum* share important phenotypic features with representatives of the genus *Desulfosporosinus*. However, at present the genus Desulfotomaculum is phylogenetically and phenotypically quite heterogenous and probably harbors numerous misclassified species. Hence, the distinction of this genus from other related taxa based on genus-specific traits is difficult and awaits taxonomic rearrangement of the species currently comprising the genus *Desulfotomaculum*.

Phenotypic Characteristics

In Table 1, the related genera *Desulfitobacterium*, *Desulfosporosinus*, *Dehalobacter* and *Syntrophobotulus* are compared, and several important distinguishing traits are listed. Phenotypic characteristics that are common to all representatives of this group include a strict anaerobic metabolism, the Gram-type positive cell wall structure, and a rod-shaped morphology. Ellipsoidal spores causing slight swelling of the cells, motility by means of flagella, and cell wall surface layers are at least present in most tested species. Gram staining depends on the strain or species but in most cases is negative. The G+C content of DNA among species of this group is in the range 45–49 mol%. In all species analyzed so far, LL-diaminopimelic acid (LL-dpm) was identified as the diagnostic diamino acid of the cell wall peptidoglycan. With one exception in all analyzed species LL-diaminopimelic acid (LL-dpm) was identified as the diagnostic diamino acid of the cell wall peptidoglycan. MK-7 represents the major menaquinone of members of the genera *Desulfitobacterium* and *Desulfosporosinus*. Physiological traits common to the genera *Desulfitobacterium* and *Desulfosporosinus* are fermentation of pyruvate, incomplete oxidation of substrates to acetate, and utilization of thiosulfate as terminal electron acceptor.

Representatives of the genus *Desulfosporosinus* can be easily distinguished from the species of other related genera by their ability to grow autotrophically with H_2, fermentation of lactate, and utilization of sulfate as terminal electron acceptor. In contrast, the hallmark feature of members of the genus *Desulfitobacterium* is their ability to use a wide spectrum of chlorinated aliphatic and aromatic compounds for reductive dehalogenation. Still unclear, however, is whether this metabolic potential is really restricted to this genus. Most representatives

Table 1. Differential characteristics of the phylogenetically related genera *Desulfitobacterium*, *Desulfosporosinus*, *Dehalobacter* and *Syntrophobotulus*.

Characteristic	*Desulfitobacterium*	*Desulfosporosinus*	*Dehalobacter*	*Syntrophobotulus*
No. of species	4[a]	3	1	1
Morphology				
Cell shape	Straight or slightly curved rods	Curved rods, occasionally filaments	Straight rods with tapered ends	Slightly curved rods
Cell diameter (μm)	0.5–0.7	0.4–1.1	0.3–0.5	0.5
Cell length (μm)	2–6	2–5	2–3	2.5–3.5
Surface layer	(+)[b]	+	+	nr
Spores	Ellipsoidal, terminal	Ellipsoidal, subterminal to terminal	None	Ellipsoidal, terminal
Flagellation	Lateral or polar or none	Polar to subpolar or lateral or none	Lateral	None
Metabolism				
Autotrophic growth with H_2	–	+	–	–
Substrate oxidation type	Incomplete	Incomplete	Nr	Incomplete
Fermentation of lactate	–	+	–	–
Dehalorespiration	+	(+)[c]	+	Nr
Electron donors used				
H_2 + acetate	D	+	+	–
Formate	+	+	–	–
Acetate	–	–	–	–
Pyruvate	+	+	–	–
Lactate	+	+	–	–
Butyrate	D	+	–	–
Yeast extract	D	+	–	–
Electron acceptors used				
Nitrate	D	–	–	–
Sulfur	+	+	–	–
Sulfite	D	+	–	–
Thiosulfate	+	+	–	–
Sulfate	–	+	–	–
Mn(IV)	(+)[d]	(+)[c]	–	Nr
PCE (tetrachlorethene)	D	–	+	Nr
Chemotaxonomy				
Diagnostic diamino acid	(LL-Dpm)[b]	LL-Dpm	LL-Dpm	Nr
Major menaquinone	(MK-7)[e]	MK-7	MK-7, MK-8	MK-9
Major cytochrome	(type c)[f]	(type b)[g]	type b	None
DNA mol% G+C	46–49	42–47	45	47

Symbols: Nr, not reported; +, positive in most strains; –, negative in most strains; and D, different reaction among strains.

Characteristics given in parenthesis were determined only for a few strains.

Abbreviations: PCE, tetrachlorethylene; Dpm, diaminopimelic acid; and MK-7, -8 and -9, menaquinones with 7, 8 and 9 isoprene units, respectively.

[a]The species epithets *Desulfitobacterium hafniense* and *Dba. frappieri* are subjective synonyms (Niggemyer et al., 2001) and hence are considered as one species in this table.

[b]Data are only available for *Desulfitobacterium* sp. strain PCE1 (Gerrites et al., 1996) and *Dba. dehalogenans* DSM 1916[T] (P. Schumann, DSMZ; personal communication).

[c]Data are only available for the reduction of PCE by *Desulfosporosinus meridiei* and *Dsp. orientis*.

[d]Data are only available for *Desulfitobacterium hafniense* and *Dba. metallireducens*.

[e]Date are only available for the *Desulfitobacterium hafniense* strains DCB-2[T] (Hippe et al., 1997) and TCP-A (Breitenstein et al., 2001).

[f]Data are only available for *Desulfitobacterium* sp. strain PCE1 (Gerrite et al., 1996) and *Desulfitobacterium hafniense* (Christiansen and Ahring, 1996; Gerritse et al., 1999).

[g]Data are only available for *Desulfosporosinus orientis* and *Desulfosporosinus* sp. DSM8344.

Data from The Genus *Desulfotomaculum*, Utkin et al. (1994), Friedrich et al. (1996), Sanford et al. (1996), Newman et al. (1997), Holliger et al. (1998), van de Pas et al. (2001), Niggemyer et al. (2001), Robertson et al. (2001), Finneran et al. (2002), and Stackebrandt et al. (2003).

of other genera, including *Desulfosporosinus* species, were not routinely tested for this characteristic and therefore this ability may have escaped attention. Nevertheless, *Desulfitobacterium* species seem to have a wider spectrum of electron acceptors and *Desulfosporosinus* species seem to have a wider spectrum of utilizable electron donors.

The Genus *Desulfitobacterium*

Habitat

Members of the genus *Desulfitobacterium* were mainly isolated from contaminated environmental sites from around the world. Habitats include soil, freshwater sediment, compost soil, and sewage sludge. Independent of the environmental site, maintenance of anoxic conditions seems to be the most important prerequisite for the proliferation of these bacteria in a specific habitat. Nevertheless, the capacity among *Desulfitobacterium* species to form endospores may promote their survival also under unfavorable growth conditions and thereby contribute to a ubiquitous distribution. Although most known *Desulfitobacterium* strains were isolated from sites contaminated with halogenated pollutants or heavy metals, the distribution of this genus is obviously not restricted to those ecological niches. This was clearly demonstrated by the isolation of *Desulfitobacterium* strains from pristine freshwater sediment (*Dba. dehalogenans*; Utkin et al., 1994) or human feces (strain DP7; Van de Pas et al., 2001). In a cultivation-independent study, soil samples from various sites in Canada were tested for the presence of indigenous *Desulfitobacterium* species by the amplification of DNA with specific polymerase chain reaction (PCR) primers targeting signature regions of their respective 16S rRNA genes. Positive signals were obtained in 31 out of 48 analyzed soil samples. A contamination of the analyzed samples with heavy metals or halogenated compounds had no effect on the presence or absence of *Desulfitobacterium* cells (Lanthier et al., 2001).

Isolation

In studies describing the selective enrichment of *Desulfitobacterium* strains from environmental samples, mainly basal freshwater mineral media were used containing as electron acceptors either halogenated compounds, arsenate (Niggemeyer et al., 2001), or the humic substance analogue anthraquinone-2,6-disulfonate (AQDH; Finneran et al., 2002). Types and concentrations of halogenated compounds used for successful enrichment comprise pentachlorophenol (PCP,

3–5 mg/liter; Bouchard et al., 1996), 2,4,6-trichlorophenol (2,4,6-TCP, 100–200 μM; Breitenstein et al., 2001), 2,3-dichlorophenol (2,3-DCP, 1 mM; Sanford et al., 1996), tetrachloroethene (PCE, 100 μM; Gerritse et al., 1996), and 1,2-dichloroethane (1,2-DCA, 400 μM; De Wildeman et al., 2003). The suitable concentration of the used halogenated compound depends on its toxicity for the dehalogenating population, its solubility in water, and the toxicity of the formed end products. Prior to the isolation of pure cultures in most cases the initial enrichments were further purified by successive transfer in batch cultures or continous incubation in bioreactors.

Depending on the abundance of the *Desulfitobacterium* population in the final enrichment, several approaches may be applied for the successful isolation of pure cultures. The most common techniques include serial dilution to extinction, soft agar shake cultures, or plating on agar media under selective or nonselective growth conditions. Anoxic conditions should be maintained during all steps of the isolation procedure because high levels of oxygen are not tolerated by most strains for longer periods. The further growth requirements of *Desulfitobacterium* strains in pure culture are generally not very fastidious, so that pre-reduced, standard freshwater mineral media supplemented with low amounts of yeast extract (0.1–0.2%) and suitable substrates are in most cases adequate for cultivation. Note, however, that a requirement for vitamin K1 in the growth medium was reported for the *Desulfitobacterium* strain DCA1 (De Wildeman et al., 2003). Isolation media should contain a buffer system (e.g., carbonate-CO_2) to keep the pH at a circumneutral value, especially if halogenated compounds are used as electron acceptors. Otherwise, HCl formed by reductive dechlorination could lead to a decrease of medium pH. Most *Desulfitobacterium* strains are not sensitive to sodium sulfide as a reductant, but several strains belonging to this phylogenetic group are inhibited by high sulfide concentrations in the medium (The Genus *Desulfotomaculum* in this Volume). Therefore, keeping the concentration of sulfide in the medium as low as possible and if necessary using dithionite (15–30 mg/liter) as an additional reductant is recommended. To monitor the redox status of the medium, adding the indicator resazurin prior to autoclaving is useful. Resazurin is pink when the redox potential is above –50 mV and colorless when the redox potential is below –110 mV (i.e., highly reduced conditions). After inoculation, cultures are normally incubated at 30–37°C for 1–5 days in the dark.

Nonselective growth media suitable for the cultivation and maintenance of most *Desulfitobacterium* strains can be found for example in the

Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; German collection of microorganisms and cell cultures) [{www.dsmz.de/media}{catalogue of strains}]. The procedure for preparing a general-purpose *Desulfitobacterium* medium is given below.

DSMZ Medium 720

NH_4Cl	1.00 g
NaCl	0.10 g
$MgCl_2 \cdot 6H_2O$	0.10 g
$CaCl_2 \cdot 2H_2O$	0.05 g
$K_2HPO_4 \cdot 3H_2O$	0.40 g
Trace element solution (see below)	1.00 ml
Selenite-tungstate solution (see below)	1.00 ml
Resazurin	0.50 mg
Yeast extract	1.00 g
$NaHCO_3$	2.60 g
Vitamin solution (see below)	10.0 ml
Na-pyruvate	2.50 g
$Na_2S_2O_3 \cdot 5H_2O$	1.25 g
$Na_2S \cdot 9H_2O$	0.30 g
Distilled water	1000 ml

Dissolve ingredients (except bicarbonate, vitamins, pyruvate, thiosulfate and sulfide), boil medium for 3 min, and then cool to room temperature under 80% N_2 + 20% CO_2. Add solid bicarbonate and adjust pH to 7.0. Dispense under the same gas mixture in anoxic vessels and autoclave. Add vitamins (sterilized by filtration), pyruvate, thiosulfate and sulfide from sterile, anoxic stock solutions prepared under N_2.

Trace Element Solution

Na_2-EDTA	0.50 g
$FeCl_2 \cdot 4H_2O$	1.50 g
$ZnCl_2$	70.00 mg
$MnCl_2 \cdot 4H_2O$	100.00 mg
H_3BO_3	6.00 mg
$CoCl_2 \cdot 6H_2O$	190.00 mg
$CuCl_2 \cdot 2H_2O$	2.00 mg
$NiCl_2 \cdot 6H_2O$	24.00 mg
$Na_2MoO_4 \cdot 2H_2O$	36.00 mg
Distilled water	990 ml

First dissolve EDTA and adjust pH to 6.5 with KOH, then add minerals. Adjust final pH to 7.0 with KOH.

Selenite-tungstate Solution

NaOH	0.50 g
Na_2SeO_3	3.00 mg
$Na_2WO_4 \cdot 2H_2O$	4.00 mg
Distilled water	1000 ml

Vitamin Solution

Biotin	2.0 mg
Folic acid	2.0 mg
Pyridoxine-HCl	10.0 mg
Thiamine-HCl $\cdot$ 2H$_2$O	5.0 mg
Riboflavin	5.0 mg
Nicotinic acid	5.0 mg
D-Ca-pantothenate	5.0 mg
Vitamin B_{12}	0.10 mg
p-Aminobenzoic acid	5.0 mg
Lipoic acid	5.0 mg
Distilled water	1000 ml

Identification

A definite affiliation of isolates to the genus *Desulfitobacterium* is in most cases possible by the distinguishing characteristics presented in Table 1 (see the section Common Traits and Differentiation in this Chapter). Currently, this genus comprises validly published descriptions of the following five species: *Dba. dehalogenans* (Utkin et al., 1994), *Dba. hafniense* (Christiansen and Ahring, 1996), *Dba. frappieri* (Bouchard et al., 1996), *Dba. chlororespirans* (Sanford et al., 1996), and *Dba. metallireducens* (Finneran et al., 2002). The type species of the genus is *Dba. dehalogenans*. Recently, genomic and phenotypic evidence showed that the type strains of the species *Dba. hafniense* and *Dba frappieri* are very closely related and should be united in one species (Niggemyer et al., 2001). *Desulfitobacterium hafniense* is the oldest legitimate epithet of this taxon and hence *Dba. frappieri* is a later heterotypic synonym of *Dba. hafniense*. Consequently, in the text of this chapter all strains that have been affiliated in the literature to *Dba. frappieri* will be referred to as *Dba. hafniense*, which has priority.

A reliable identification of distinct species within this genus is more complex and requires a profound polyphasic characterization based on phenotypic and molecular methods. The difficulties inherent to the classification of *Desulfitobacterium* strains arise from their metabolic plasticity which prevents a clear cut assignment of isolates to distinct species on the basis of phenotypic characteristics. This is most evident in *Dba. hafniense*. A comparison of the characteristics of strains which were at least tentatively affiliated to this species allows a good estimate of the intraspecific diversity possible in species of this genus. For instance, the *Dba. hafniense* strain DP7 was shown incapable of dehalogenating chlorinated compounds (Van de Pas et al., 2001), whereas strain PCE-S was shown incapable of utilizing sulfite or thiosulfate as electron acceptors (Miller et al., 1998). The lack of these features in *Desulfitobacterium* strains is unexpected because these were thought to represent hallmark traits of this genus. Consequently, molecular methods like the comparative analysis of 16S rRNA gene sequences and DNA-DNA hybridization experiments are necessary for the unambiguous identification of isolates.

A list of distinguishing traits useful for the differentiation of *Desulfitobacterium* species can be found in Table 2.

Morphology

Members of the genus *Desulfitobacterium* have generally a straight to slightly curved rod-shaped

Table 2. Main morphological and physiological characteristics of *Desulfitobacterium* species.

Characteristic	*Dba. Dehalogenans*	*Dba. hafniense*[a]	*Dba. chlororespirans*	*Dba. metallireducens*
No. of strains	1	3	1	1
Gram stain	Positive	Negative	Negative	Positive
Spores	None	Ellipsoidal, terminal	Ellipsoidal, terminal	None
Motility	+	D	+	−
Temperature optimum (°C)	38	37	37	30
Growth on solid medium (1.5% agar)	−	+	+	+
Fermentation of pyruvate	+	+	+	−
Electron donors used				
H_2 + acetate	+	−	+	−
Formate	+	+	+	+
Acetate	−	−	−	−
Pyruvate	+	+	+	+
Lactate	+	+	+	+
Butyrate	+	D	+	+
Succinate	Nr	D	−	Nr
Ethanol	+	D	Nr	+
Glycerol	Nr	−	Nr	−
Glucose	Nr	−	Nr	−
Electron acceptors used				
Fumarate	+	+	−	−
Nitrate	+	D	−	−
Sulfur	+	+	+	+
Sulfite	+	+	+	−
Thiosulfate	+	+	+	+
Sulfate	−	−	−	−
As(V)	−	+	Nr	Nr
Fe(III)	Nr	+	Nr	+
Mn(IV)	Nr	+	Nr	+
2-chlorophenol	−	−	−	Nr
2,4-dichlorophenol	+	+	−	Nr
2,4,6-trichlorophenol	+	+	+	Nr
Pentachlorophenol	+	+	−	Nr
3-Cl-4-OHPA	+	D	+	+
PCE	−	D	−	+
DNA mol% G+C	46	47	49	Nr

Symbols and abbreviations: Nr, not reported; +, positive in most strains; −, negative in most strains; and D, different reaction among strains; 3-Cl-4-OHPA, 3-chloro-4-hydroxyphenylacetate; and PCE, tetrachloroethene.

Abbreviations: 3-Cl-4-OHPA, 3-chloro-4-hydroxyphenylacetate; and PCE, tetrachloroethene.

[a]The species epithets *Desulfitobacterium hafniense* and *Dba. frappieri* are subjective synonyms (Niggemyer et al., 2001) and hence are considered as one species. Only the strains DCB-2[T], PCP-1, and GBFH were considered in this table because their affiliation to the species *Dba. hafniense* was confirmed by DNA-DNA hybridization.

Data from Utkin et al. (1994), Christiansen and Ahring (1996), Bouchard et al. (1996), Sanford et al. (1996), Dennie et al. (1998), Niggemyer et al. (2001), van de Pas et al. (2001), and Finneran et al. (2002).

morphology. The cell dimensions are 0.5–0.7 μm × 2–6 μm. A phase contrast micrograph of *Dba. dehalogenans* DSM 9161[T] is shown in Fig. 2.

The Gram staining depends on the species and is positive in *Dba. dehalogenans* and *Dba. metallireducens* but negative in *Dba. hafniense* and *Dba. chlororespirans*. To date, ellipsoidal and terminal spores that slightly swell the cell were detected only in *Desulfitobacterium* species that stain Gram negative. In general, the tendency of *Desulfitobacterium* strains to form spores is less pronounced than in cultures of *Desulfosporosinus* species and the frequency of spore-forming cells is low.

Flagellation is variable and strain specific. Most strains are motile by means of polar or lateral flagella, but the type strain of *Dba. metallireducens*, 853-15A[T], and the *Dba. hafniense* strain PCP-1 are nonmotile. Interestingly, the type strain of *Dba. hafniense*, DCB-2[T], is motile by means of 1–2 polar flagella, whereas the *Dba. hafniense* strain GBFH possesses 2–5 lateral flagella.

With the exception of the *Dba. dehalogenans* strain JW/IU-DC1[T], all described strains form colonies on solid agar media. Colonies on agar surfaces are white, translucent, circular to slightly irregular and smooth. They reach several mm in diameter.
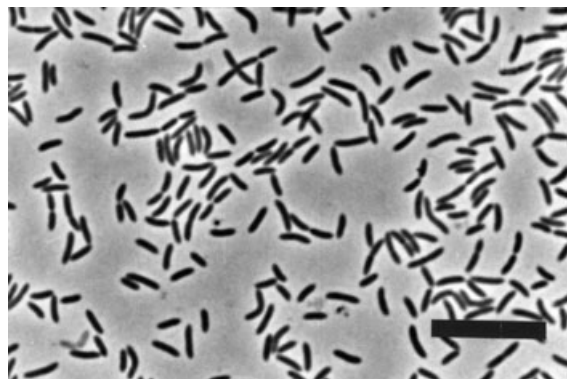
Fig. 2. Phase contrast micrograph of *Dba. dehalogenans* DSM 9161[T]. Bar = 10 μm.

Physiology and Chemotaxonomy

In Table 2, several physiological characteristics helpful for the differentiation of *Desulfitobacterium* species are listed.

A comparison of whole cell fatty acid patterns of the type strains of the genus *Desulfitobacterium* can be found in Table 3. In general, the fatty acid patterns of representatives of this genus are rather complex and comprise several compounds that were not identified by the MIDI system (Microbial ID, Inc., Newark, DE). The following fatty acids were found to be dominating in most strains: 14:0, 16:1 *cis*9, 16:0, and 18:1 *cis*11. The fatty acid patterns of the *Dba. hafniense* strains DSM 10664[T] and DSM 12420 (formerly representing *Dba. frappieri*) were very similar, whereas the patterns of the phylogenetically distinct species *Dba. dehalogenans* and *Dba. metallireducens* are clearly different. Hence, a differentiation of species within this genus by means of fatty acid patterns seems to be possible. In *Dba. dehalogenans* the three characteristic fatty acids are 16:0, 14:0, and 16:1 *cis*9; in *Dba. hafniense* 18:1 *cis*11 dma, 18:1 *cis*11, and 16:1 *cis*9; in *Dba. chlororespirans* 16:0, 16:1 *cis*9, and 14:0; and in *Dba. metallireducens* 14:0, 16:1 *cis*9 dma, and an unidentified fatty acid with an equivalent chain length (ECL) of 13.52.

Molecular Methods

The determination and comparative analysis of 16S rRNA gene sequence data is a valuable method for the identification of *Desulfitobacterium* isolates. Studies on several *Dba. hafniense* strains have clearly shown that methods based on the analysis of DNA are more reliable for the affiliation of strains to distinct *Desulfitobacterium* species than physiological or biochemical tests (Van de Pas et al., 2001). However, the diversity of multiple 16S rRNA operons in *Desulfitobacterium* strains caused in the past some confusion about their correct phylogenetic placement. In the *Dba. hafniense* strains PCP-1 (Lévesque et al., 1997) and GBFH (Niggemyer et al., 2001) at least three distinct rRNA operons could be detected. The various 16S rRNA operons of *Desulfitobacterium* strains can differ by the size of an insert between nucleotide position 75 and 94 (*Escherichia coli* numbering). The standard size of this region is 22 nucleotides, but in distinct operons it can reach 139 nucleotides (Van de Pas et al., 2001). The presence of multiple operons among *Desulfitobacterium* species was overlooked for several years because of the cloning of PCR products leading to the subsequent sequencing of just one 16S rRNA gene. In contrast, the direct sequencing of amplified 16S rRNA gene fragments, which is now a standard method, often reveals multiple operons by clearly defined unreadable regions which are caused by overlapping sequence stretches. To avoid a bias in the reconstruction of phylogenetic trees it is advisable to completely remove the 5' terminal hypervariable region of *Desulfitobacterium* 16S rRNA gene sequences (*E. coli* positions 1 to 94) prior to the calculation of distance matrices. A phylogenetic dendrogram illustrating the intrageneric relationship of most known *Desulfitobacterium* strains is shown in Fig. 3.

In Fig. 3, the large number of strains which can be affiliated to the species *Dba. hafniense* is shown. All strains within this cluster share 16S rRNA sequence similarity values (*E. coli* nucleotide positions 95–1521) above 99%. The affiliation of the strains PCP-1 (formerly *Dba. frappieri*) and GBFH to the species *Dba. hafniense* was confirmed by DNA-DNA hybridization experiments with the type strain DCB-2[T] (Niggemeyer et al., 2001). DNA-DNA hybridization data exist also for the two *Dba. hafniense* strains TCE1 and DP7 (83%; Van de Pas et al., 2001).

On the other hand, DNA-DNA binding values of *Dba. hafniense* DCB-2[T] with the phylogenetically distinct strains *Dba. chlororespirans* Co23[T] and *Dba. dehalogenans* JW/IU-DC-1[T] were only 64.3% and 43.9%, respectively (Niggemyer et al., 2001). The threshold value for strains belonging to one species is 70% (Wayne et al., 1987).

Hence, a comparison of both genetic approaches within this clade of bacteria justifies the conclusion that strains which share 16S rRNA sequence similarity values above 99% probably belong to the same species, whereas values below 98% clearly indicate the affiliation of strains to separate species. However, this assumption is restricted to the known *Desulfitobacterium* strains and may not holds true for other taxa.

Table 3. Fatty acid compositions of the type strains of *Desulfitobacterium* species.[a]

ECL	Fatty acid[b]	*Dba. dehalogenans*[c] DSM 9161[T]	*Dba. hafniense*[c] DSM 10664[T]	*Dba. frappieri*[c] DSM 12420[T]	*Dba. chlororespirans*[c] DSM 11544[T]	*Dba. metallireducens*[c] DSM 15288[T]
13.52	Unknown	0.8	—	—	4.9	19.4
13.62	*i*-14:0	—	—	—	—	1.1
13.81	Unknown	1.3	1.9	1.8	2.0	1.5
14.00	14:0	22.8	4.0	4.9	11.4	16.3
14.42	Unknown	—	—	—	—	0.5
14.63	*i*-15:0	—	—	—	—	0.9
14.71	*ai*-15:0	—	—	—	—	0.8
14.77	Unknown	1.0	—	—	—	5.2
14.79	15:1 *c*7	—	0.6	0.6	0.6	—
14.86	15:1 *c*9	—	0.3	0.4	0.3	1.1
14.95	16:0 ald	2.7	0.9	0.8	1.1	0.8
15.00	15:0	0.7	0.4	0.5	0.6	2.9
15.33	Unknown	—	—	—	—	0.5
15.41	Unknown	—	—	—	—	0.6
15.47	15:0 dma	—	—	—	—	1.1
15.77	16:1 *c*7	5.0	7.8	8.4	8.1	3.5
15.81	16:1 *c*9	10.8	11.3	13.0	12.2	6.4
15.90	16:1 *c*11	0.5	1.8	2.1	1.5	1.4
16.00	16:0	25.6	10.9	10.6	14.0	4.4
16.24	16:1 *c*7 dma	—	0.2	0.2	0.3	3.7
16.28	16:1 *c*9 dma	1.6	0.5	0.6	0.7	12.7
16.38	Unknown	—	0.1	0.2	0.2	1.1
16.47	16:0 dma	5.4	5.4	4.7	5.5	4.3
16.76	Unknown	0.9	0.7	0.7	0.6	—
16.79	17:1 *c*9	—	—	—	—	0.7
16.80	Unknown	2.5	3.1	2.9	2.2	—
16.87	17:1 *c*11	—	0.7	0.6	0.4	0.6
16.97	18:0 ald	—	1.9	1.2	1.0	—
17.25	18:1 dma	—	0.3	0.3	0.2	1.6
17.33	Unknown	—	0.4	0.5	0.2	0.8
17.47	17:0 dma	—	0.8	0.5	0.3	—
17.76	18:1 *c*9	2.7	3.5	4.0	3.5	0.6
17.82	18:1 *c*11	6.0	12.1	13.6	10.6	0.5
17.92	18:1 *c*13	—	0.5	0.6	0.3	—
18.00	18:0	2.0	4.4	3.8	3.1	0.7
18.22	18:1 *c*9 dma	1.9	4.0	3.9	2.5	1.2
18.28	18:1 *c*11 dma	4.4	13.1	12.7	7.5	0.9
18.39	Unknown	—	0.5	0.5	0.2	—
18.47	18:0 dma	0.7	4.9	2.9	1.9	0.7
19.77	20:1 *c*11	—	1.2	1.2	0.6	—
19.83	20:1 *c*13	—	0.7	0.7	0.4	—

Symbols and abbreviations: ECL, equivalent chain length; dma, dimethylacetal; *i*, iso; ald, aldehyde; *c*, cis; and —, not present or below detction limit.
[a]The strains DSM 9161[T], DSM 10664[T], DSM 12420[T], and DSM 11544[T] were grown in DSM medium 720 for 24h prior to analysis, whereas DSM 15288[T] was cultured in DSM medium 838 for 48h.
[b]Unknown fatty acids could not be identified with the Microbial Identification (Microbial ID) standard software package (Sasser, 1990).
[c]Data are percent total fatty acids. The three most dominating fatty acids of each strain are in bold. Fatty acids that were detected only in trace amounts (<0.5%) in all analyzed strains were not considered in this table.

The automated RiboPrinter system (DuPont Qualicon) offers a fast and reliable approach for the identification of *Desulfitobacterium* strains by analysis of genomic DNA (Bruce, 1996). In Fig. 4, riboprint patterns of all *Desulfitobacterium* type strains generated with the restriction enzyme *Eco*RI are shown. The resulting riboprints were specific for each tested strain. The riboprints of the *Dba. hafniense* strains DSM 10664[T] and DSM 12420 (formerly representing *Dba. frappieri*) were most similar, but clearly distinct from all other patterns. Consequently, the diversity of patterns correlated well with the phylogenetic relationships of these strains.

For the detection of *Desulfitobacterium* species in the environment, specific PCR primers were developed on the basis of 16S rRNA gene sequences. Specific primer sets enabling nested

PCR were designed for *Dba. dehalogenans* (Fantroussi et al., 1997) and *Dba. hafniense* (formerly *Dba. frappieri*; Lévesque et al., 1997). The amplification of *Desulfitobacterium* DNA with the primer sets binding to the terminal regions of the 16S rRNA genes results in PCR fragments with a size of 1199 nucleotides in *Dba. dehalogenans* and 1080 nucleotides in *Dba. hafniense*. The obtained PCR products can be used as template for the amplification of internal fragments in a second round PCR reaction. By application of the nested PCR approach, the sensitivity for the detection of *Desulfitobacterium* cells in soil could be increased several times compared to the one-step PCR. The detection limit under optimal conditions was approximately 10^3 cells/g of soil. A further advantage of the nested PCR approach is that the risk of false positive signals is reduced. This approach was successfully used for the monitoring of *Desulfitobacterium* cells in laboratory

enrichment cultures or soil (Lanthier et al., 2000; Lanthier et al., 2001).

An interesting alternative procedure for the detection of *Desulfitobacterium* cells in the environment was presented by Christiansen and Ahring (1996). They derived calibrated antibody probes from rabbit antisera against *Dba. hafniense* DCB-2^T. Cells which have bound the specific antibody could be visualized by indirect immunofluorescence. The advantage of this method is that a direct count of cells in environmental samples is possible. However, a careful control of the specificity of the antibody has to be performed to avoid false positive signals by crossreactivity, a frequently encountered problem in the application of specific antibodies.

A further method for the direct visualization of single cells in environmental samples was developed by Lanthier et al. (2002). They designed specific fluorescently labelled oligonucleotide probes targeting distinct regions of the respective 16S rRNA molecules of *Desulfitobacterium. hafniense* and *Dba. chlororepirans*. With this method the intensity of the hybridization signal depends largely on the intracellular content of ribosomal RNA. Consequently, this has the advantage that only metabolically active cells are detected using epifluorescence microscopy, while resting cells remain unseen owing to their low content of ribosomal RNA.
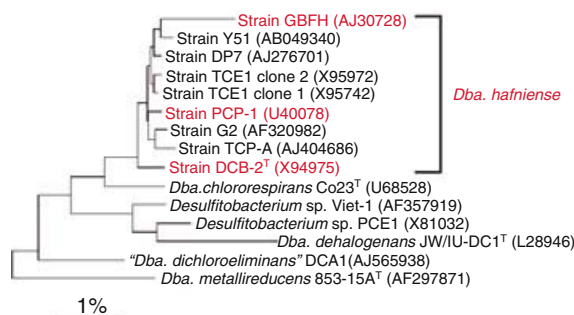
Fig. 3. Phylogenetic dendrogram based on partial 16S rRNA gene sequences (nucleotides 95 to 1521, *E. coli* numbering) showing the intrageneric relationship of *Desulfitobacterium* strains. Strains for which the affiliation to the species *Dba. hafniense* was confirmed by DNA-DNA hybridization are shown in red. The GenBank/EMBL accession number for each sequence is shown in parenthesis. The tree was reconstructed using the ARB program package (http://www.arbhome.de). It is derived from a distance matrix reconstructed by using the neighbor joining method of Saitou and Nei (1987). Phylogenetic distances were calculated as described by Felsenstein (1982). The sequence of *Desulfosporosinus orientis* (accession no. [Y11570]) was used to define the root (not shown). Scale bar represents 1% estimated sequence divergence.

Preservation

Stock cultures of most *Desulfitobacterium* strains can be kept for about one month at 4°C without loss of viability. For the intermediate storage of cultures, freezing in anoxic medium supplemented with 50% glycerol may be suitable. However, for the long-term storage, lyophilization is recommended. Some *Desulfitobacterium* strains may be sensitive to the freeze drying process and display low survival rates after lyophilization. For the long-term preservation of these strains, freezing in liquid nitrogen is a suitable method using suspensions of cells in freshly prepared medium supplemented with 5% of the cryoprotectant dimethyl sulfoxide (DMSO). Detailed descriptions for the lyophilization and deep

Fig. 4. Diversity of riboprint patterns of *Desulfitobacterium* type strains generated with *Eco*RI. The similarity dendrogram was generated using Bionumerics software (Applied Maths).
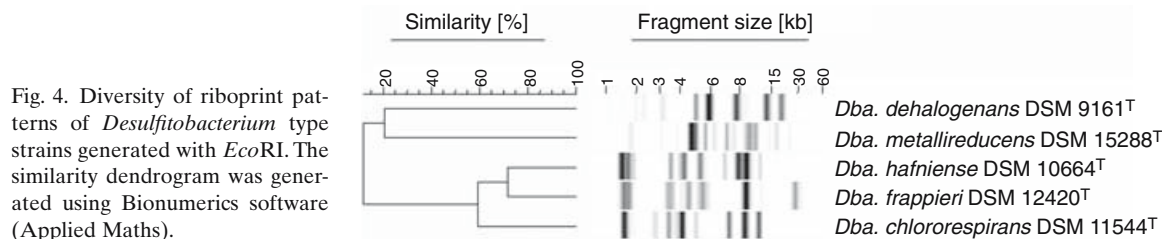
freezing of anaerobes can be found in the contribution of Hippe (1991).

The Genus *Desulfosporosinus*

Habitat

Members of the genus *Desulfosporosinus* were mainly isolated from water-saturated soil environments and freshwater sediments that are usually anoxic. The *Desulfosporosinus* strain 343 (DSM 8344) was isolated from permafrost deposits of an ancient river bed in Kolymskaya Lowland, Russia. Presumably, the cells of this strain were not active in situ but survived in the form of spores for a few thousand years in the permafrost soil (Vainshtein et al., 1995).

Isolation

In cultures of many *Desulfosporosinus* strains, a significant number of sporeforming cells is frequently observed. The formation of heat-resistant endospores makes it possible to use essentially the same selective enrichment and isolation procedures and media for these strains as described for sporeforming sulfate reducers of the genus *Desulfotomaculum* (The Genus *Desulfotomaculum* in this Volume). If necessary, nonsporeforming sulfate reducers which frequently outcompete members of the genus *Desulfosporosinus* in samples of permanently anoxic habitats can be eliminated by pasteurization of samples at 80°C for 10–20 min.

The species *Dsp. auripigmenti* was selectively enriched from a mixed culture of sulfate reducers obtained from a contaminated lake sediment by replacing sodium sulfate with 10 mM dibasic sodium arsenate as electron acceptor in the used freshwater mineral medium. Lactate (20 mM) served as carbon and energy source and cysteine was used as reducing agent and source of reduced sulfur. Growth and subsequent reduction of arsenate became visible by the formation of a yellow precipitate, determined to be As_2S_3 (Newman et al., 1997). A purification of the isolate responsible for arsenate reduction was achieved by successive colony transfers in agar shake tubes.

Most strains of *Desulfosporosinus*, however, were enriched and isolated under nonselective growth conditions by serial dilution of samples of anoxic soil or sediment in agar deeps or liquid media. For the purification of isolates, serial dilution to extinction, soft agar shake cultures, or plating on agar media are suitable methods. Anoxic conditions should be maintained during all steps of the isolation procedure because high levels of oxygen are not tolerated by vegetative cells of *Desulfosporosinus* for longer periods. For

the same reason, media used for isolation should be pre-reduced. Suitable reductants are sodium sulfide, cysteine, ascorbate and thioglycollate, or sodium dithionite. *Desulfosporosinus orientis* among other Gram-positive sporeforming sulfate reducers occasionally shows poor growth in media that have been reduced with sodium sulfide. Certain impurities in the commercially available Na_2S likely have an inhibitory effect on the growth of these strains (The Genus *Desulfotomaculum* in this Volume). For the cultivation of these microorganisms, alternative reductants should be used or the amount of sulfide should be reduced to a minimum. If a low amount of sulfide is used (<100 mg/liter), the addition of 10–20 mg of sodium dithionite per liter of medium shortly before inoculation (e.g., from a 5% [w/v] solution, freshly prepared under N_2, and filter-sterilized) often stimulates growth of sulfate reducers at the beginning.

All known members of this genus grow well and occasionally sporulate in the DSMZ medium 641 DSMZ catalogue of strains (http://www.dsmz.de/media) which has been modified by replacing malate and pyruvate with sodium lactate (2.5 g/liter). The procedure for preparing this medium is given below.

Modified DSMZ Medium 641

NH_4Cl	1.0 g
Na_2SO_4	2.0 g
Na-thiosulfate · $5H_2O$	1.0 g
$MgSO_4$ · $7H_2O$	1.0 g
$CaCl_2$ · $2H_2O$	0.1 g
KH_2PO_4	0.5 g
Resazurin	0.5 mg
Trace element solution	
(see *Desulfitobacterium* medium above)	1.0 ml
Yeast extract	1.0 g
Na-lactate	2.5 g
$NaHCO_3$	2.0 g
Vitamin solution	
(see *Desulfitobacterium* medium above)	10.0 ml
Na_2S · $9H_2O$	75.0 mg
Distilled water	1000 ml

Dissolve ingredients (except bicarbonate, vitamins and sulfide), boil medium for 3 min., and then cool to room temperature under 100% N_2. Dispense medium under N_2 gas atmosphere in tubes or bottles and autoclave. Complete the medium by adding vitamins (sterilized by filtration), sulfide (from sterile, anoxic stock solutions prepared under N_2) and bicarbonate (from a sterile, anoxic stock solution prepared under 80% N_2 and 20% CO_2). Adjust pH of the completed medium if necessary to about 7.0–7.2.

Identification

The genus *Desulfosporosinus* is well defined on the basis of physiological and chemotaxonomical traits. However, a differentiation from other genera of Gram-positive, sporeforming sulfate

reducers, especially *Desulfotomaculum* species, is often not unambiguously possible on the basis of a physiological characterization. For instance, both species *Desulfotomaculm ruminis* and *Desulfosporosinus orientis* are morphologically and nutritionally very similar but phylogenetically only distantly related. Thus, a reliable identification of isolates should always be preceded by a phylogenetic analysis of DNA sequence data. The genus *Desulfosporosinus* comprises currently three different species: *Dsp. orientis* (Campbell and Postgate, 1965; Stackebrandt et al., 1997), *Dsp. meridiei* (Robertson et al., 2001), *Dsp. auripigmenti* (Newman et al., 1997; Stackebrandt et al., 2003).

The type species of the genus is *Dsp. orientis*, which currently includes three different strains. However, the description of this taxon is exclusively based on the type strain Singapore I[T] (DSM 765[T]). The mesophilic strain 343 (DSM 8344) isolated from ancient permafrost soil in Russia was classified by Vainshtein et al. (1995) as *Dsp. orientis* solely on the basis of a phenotypic characterization. Recently published phylogenetic data based on 16S rRNA gene sequence information (R. F. Rosenweig et al., unpublished results) and DNA-DNA hybridization experiments (Stackebrandt et al., 1997) are contradictory to this assumption and clearly indicate that strain 343 represents a novel species within the genus *Desulfosporosinus*. In contrast, the identification of strain Ulu Singapore II (DSM 7439) as *Dsp. orientis* is supported by 16S rRNA gene sequence analysis and the high similarity between riboprint patterns of this strain and the type strain Singapore I[T] (Stackebrandt et al., 2003). However, for the definite affiliation to *Dsp. orientis*, a DNA-DNA hybridization experiment with the type strain would be necessary. Likewise, *Desulfosporosinus* the description of auripigmenti, is based only on a single strain. Consequently, the intraspecific diversity (which can be expected among species of this genus) could be only studied with strains of one species. A knowledge of the possible intraspecific variability within a genus is important to estimate the value of distinct taxonomic traits for the identification of species. In general, the phenotypic variability of a taxon depends on the range of genomic diversity within clonal populations composing this taxon. Variable traits of *Dsp. meridiei* include nitrate reduction (3 out of 7 strains, type strain negative) and utilization of formate (3 out of 7 strains, type strain positive), caproate (1 out of 7 strains, type strain negative), caprylate (4 out of 7 strains, type strain negative), methanol (2 out of 7 strains, type strain positive), and syringic acid (3 out of 7 strains, type strain positive).

A list of distinguishing traits useful for the differentiation of *Desulfosporosinus* species can be found in Table 4.

Morphology

Cells representing *Desulfosporosinus* strains are generally described as slightly bent or curved rods ("sausage-shaped"). The cells are 0.4–1.2 µm × 2.5–5.5 µm. Occasionally, long filament-like cells are observed. Large rods are often flexible and display a snake-like twisting motility often without forward motion.

Almost all *Desulfosporosinus* strains stain Gram-negative or Gram-variable, but *Dsp. auripigmenti* stains Gram-positive. The cytoplasm of cells in aged cultures of *Dsp. orientis* may sometimes appear granulated because of the formation of phase-dense inclusion bodies. The strains of all described species form ellipsoidal spores that slightly swell the cell (Fig. 5a). Spores are located mainly at a subterminal position; in *Dsp. auripigmenti*, frequently also terminal. The unclassified strain 343 (DSM 8344) stains Gram positive and exhibits spherical spores located at a terminal position.

An active swimming motility is in several strains restricted to the early logarithmic growth phase. In the first description of *Dsp. orientis* by Adams and Postgate (1959), the French bacteriologist A. Prevot is cited as the scientist who analyzed the flagellation type of this species. Prevot found that (sub)polar or bipolar flagellation was predominant, and cells that appeared peritrichously flagellated were rare and may represent pairs or chains of cells with bipolar flagella (". . . la forms la plus fréquent est le cil unique polaire ou para-polaire. Puis vient en deuxième position la ciliature bipolaire, enfin moins fréquent la ciliature en apparence péritriche 3 à 4 cils. Mais si on analyse minutieusement les formes à 3 ou 4 cils on s'aperçoit que ce sont des diplo-vibrions ou des chainettes de vibrions"). In a later published description of this species by Campbell and Postgate (1965), only a peritrichous flagellation type was mentioned, which is inconsistent with the early investigation of Prevot. To clarify this point the flagellation type of this strain was examined by one of the authors (SS). At a first look it actually appears that several flagella are randomly distributed on the cell surface of this strain. However, at a closer examination most cells have either lateral or subpolar to polar flagella, whereas a truly peritrichous flagellation type could not be detected (Fig. 5b). The number of flagella per cell may change with the age of the culture inasmuch as far more cells have single polar or bipolar flagella in stationary phase cultures than in young cultures, which predominantly contain cells with lateral flagellation.

Table 4. Main morphological and physiological characteristics of *Desulfosporosinus* species.

Characteristic	Dsp. orientis	Dsp. meridiei	Dsp. auripigmenti
No. of strains	2[a]	8	1
Gram stain	Negative	Negative or variable	Positive
Spores	Ellipsoidal, subterminal	Ellipsoidal, subterminal	Ellipsoidal, subterminal to terminal
Flagellation type	(Sub)polar or lateral	Lateral	None
Temperature range / optimum (°C)	?–42 / 30–37	10–37 / 28	Nr / 25–30
NaCl range	0 < 5%	0 < 4%	Nr
Fermentation of pyruvate and lactate	+	+	Nr
Electron donors used			
H$_2$ (+CO$_2$)	+	+	+
Formate	+	D (+)[b]	+
Acetate	–	–	–
Pyruvate	+	+	+
Lactate	+	+	+
Butyrate	+	+	+
Propionate	–	–	–
Succinate	–	–	–
Fumarate	+	–	–
Malate	–	–	+
Benzoate	–	–	–
Methanol	+	D (+)	–
Ethanol	+	+	+
Glycerol	–	–	+
Glucose	–	–	–
Electron acceptors used			
Fumarate	+	–	–
Nitrate	–	D (–)	–
Sulfur	+	+	+
Sulfite	+	+	+
Thiosulfate	+	+	+
Sulfate	+	+	+
As(V)	–	–	+
Fe(III)	–	–	–
Mn(IV)	–	–	–
PCE	Nr	+	Nr
DNA mol% G+C	42	47	42

Symbols and abbreviations: Nr, not reported; +, positive in most strains; –, negative in most strains; D, different reaction among strains; and PCE, tetrachloroethene.

[a]Data are solely based on the type strain Singapore I[T] (DSM 765[T]).

[b]If the reaction was variable among the tested strains, the result for the type strain is given in parenthesis.

Data from Campbell and Postgate (1959), Klemps et al. (1985), Newman et al. (1997), Robertson et al. (2001), and Stackebrandt et al. (2003).
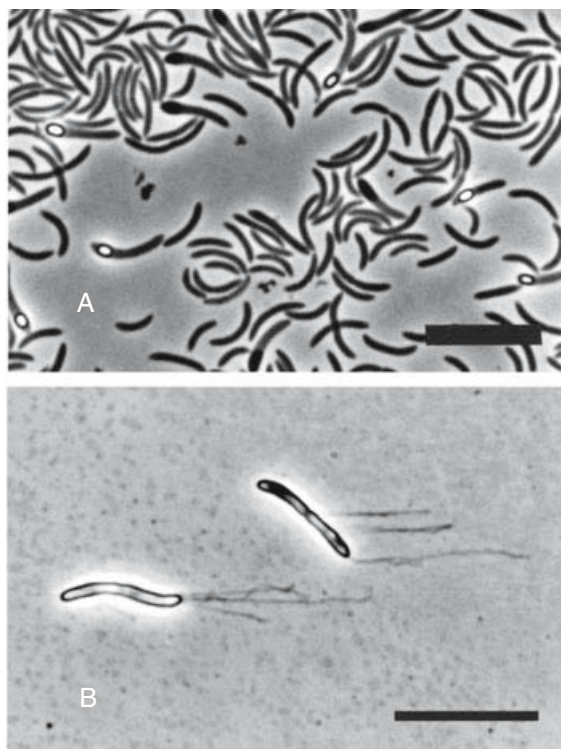
Fig. 5. Morphological characteristics of *Desulfosporosinus* species. (a) Phase contrast micrograph of a sporulating culture of *Dsp. auripigmenti* DSM 13351[T]. Bar = 10 μm. (b) Typical flagellation pattern of *Dsp. orientis* DSM 765[T] visible after staining according to the protocol of Heimbrook et al. (1989). Bar = 10 μm.

Strains of *Dsp. meridiei* are motile by means of a single subpolar or lateral flagellum. The type strain of *Dsp. auripigmenti* was described as nonmotile.

Physiology and Chemotaxonomy

Table 4 lists physiological characteristics of *Desulfosporosinus* species and Table 5 lists whole cell fatty acid patterns of the type strains of *Desulfosporosinus* species.

Compared to the fatty acid patterns of *Desulfitobacterium*, those of *Desulfosporosinus* strains are in general less complex and the variability among distinct species is less pronounced. The predominant fatty acids in most strains were 16:1 *cis*9, 16:0, and 16:0 dma. Fatty acid patterns of the type strains of *Dsp. orientis* and *Dsp. auripigmenti* were quite similar, whereas a differentiation from *Dsp. meridiei* may be possible by the low amount of the fatty acid 16:0.

A useful chemotaxonomic marker for the differentiation of *Dsp. idahoensis* from other *Desulfosporosinus* species is the detection of *meso*-diaminopimelic acid. This diamino acid has so far been detected in only the cell wall pepti-

doglycan of *Dsp. idahoensis*, whereas LL-Dpm was found to predominate in all other species of this genus.

Molecular Methods

The method of choice for the definite affiliation of isolates to the genus *Desulfosporosinus* is the determination and comparative analysis of 16S rRNA gene sequences, whereas an unambiguous identification at the species level is hardy possible solely on the basis of 16S rRNA gene sequence data. Large insertions or deletions in distinct operons of 16S rRNA genes, as found in *Desulfitobacterium* strains, were so far not detected in members of the genus *Desulfosporosinus* by the direct sequencing of PCR products. Nevertheless, the presence of multiple operons differing only by single base exchanges cannot be completely excluded since minor variations in 16S rRNA genes are not reliably detected by this approach and other methods for the detection of multiple operons have not up to now been applied to this genus. The limited value of 16S rRNA gene sequencing for the identification of *Desulfosporosinus* species is illustrated by the fact that both *Dsp. lacus* and *Dsp. idahoenis* possess almost identical 16S rRNA genes sharing a similarity value of 99.7%. Interestingly, both species were isolated from different environments and geographical sites and can be easily distinguished on the basis of phenotypic or chemotaxonomic traits. In addition, the delineation between both type strains was confirmed by a DNA-DNA binding value below 70% (63%) and clearly different riboprint patterns (In Figure 6 it is two strains of *Dsp. orientis*, DSM 765[T] and DSM 7439, with 99.7% 16S rRNA gene similarity, show similar riboprint patterns to the type strain shown that STP12 representing the tentatively novel species "Desulfosporosinus lacus", which has only 96.4% similarity to the type strain of *Dsp. orientis*. Consequently, neither 16S rRNA gene sequencing nor ribotyping alone are suitable approaches for the reliable identification of *Desulfosporosinus* species. On the other hand, the taxonomic status of the remaining *Desulfosporosinus* type strains and the separate position of the strain DSM 8344 was confirmed by DNA-DNA hybridization experiments and individual riboprint patterns (Robertson et al., 2001; Stackebrandt et al., 2003).

Preservation

The procedures for maintaining and preserving *Desulfosporosinus* strains are similar to the methods described for *Desulfitobacterium*. In

Table 5. Fatty acid compositions of the type strains of *Desulfosporosinus* species.

ECL	Fatty acid[a]	*Dsp. orientis*[b] DSM 765[T]	*Dsp. meridiei*[b] DSM 13257[T]	*Dsp. auripigmenti*[b] DSM 13351[T]
14.00	14:0	4.0	3.7	2.2
14.48	14:0 dma	—	—	—
14.63	*i*-15:0	—	—	—
14.77	Unknown	—	2.8	—
14.86	15:1 *c*9	—	1.0	—
14.95	16:0 ald	2.8	2.6	1.9
15.00	15:0	—	—	—
15.63	*i*-16:0	—	—	—
15.77	16:1 *c*7	3.0	3.5	1.5
15.81	16:1 *c*9	25.5	33.4	31.6
15.90	16:1 *c*11	0.9	1.1	—
16.00	16:0	17.4	5.8	14.2
16.24	16:1 *c*7 dma	—	1.9	—
16.28	16:1 *c*9 dma	1.8	5.6	1.7
16.47	16:0 dma	8.7	13.5	13.4
16.76	unknown	—	1.3	—
16.77	17:1 *c*8	—	0.8	1.3
16.79	17:1 *c*9	—	—	1.1
16.89	17:0 *cyc*	—	—	0.6
17.00	17:0	—	—	—
17.25	18:1 dma?	—	1.1	—
17.47	17:0 dma	—	1.3	—
17.76	18:1 *c*9	7.5	6.1	7.5
17.82	18:1 *c*11	5.6	3.3	2.5
17.92	18:1 *c*13	1.2	1.0	1.0
18.00	18:0	4.3	2.2	2.8
18.22	18:1 *c*9 dma	1.5	3.9	7.7
18.28	18:1 *c*11 dma	1.9	4.3	4.4
18.47	18:0 dma	1.1	—	2.0
19.36	19:0 *cyc* dma	—	—	—
19.77	20:1 *c*11	—	—	—
19.83	20:1 *c*13	—	—	—

Symbols and abbreviations: Please refer to footnote in Table 3.

[a]Unknown fatty acids could not be identified with the Microbial Identification (Microbial ID) standard software package (Sasser, 1990).

[b]Data are percent total fatty acids. The three most dominating fatty acids of each strain are in bold. Fatty acids that were detected only in trace amounts (<0.5%) in all analyzed strains were not considered in this table.

Adapted from S. Ramamoorthy et al. (submitted).
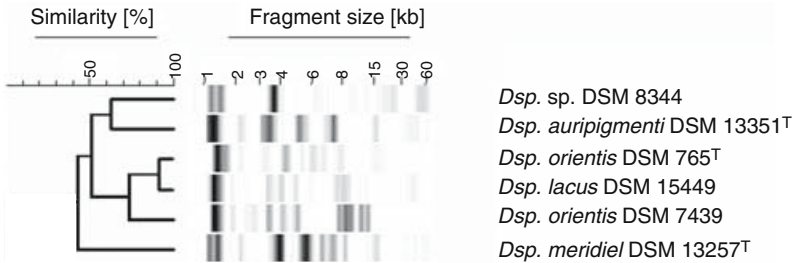
Fig. 6. Diversity of riboprint patterns of *Desulfosporosinus* type strains and related strains generated with *Eco*RI. The similarity dendrogram was generated using Bionumerics software (Applied Maths, Belgium).

addition, the pronounced tendency of some *Desulfosporosinus* strains to form spores enables the maintainance of cultures in the sporulated state (optimally as deep agar colonies). Sporulated cultures are usually stable for several months at temperatures around 4°C. The DSMZ medium 641 is suitable for the induction of sporulation in most strains. The sporulation in *Dsp. orientis* can be efficiently induced if this species is grown under sulfate limitation with hydrogen as electron donor (Cypionka and Pfennig, 1986).

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CHAPTER 1.2.25

The Genus *Desulfotomaculum*

FRIEDRICH WIDDEL

A summary of the diversity and biochemistry of bacteria that carry out a dissimilatory sulfate reduction has been given in Chapter 24. Sulfate-reducing bacteria that form heat-resistant endospores are classified in one genus, *Desulfotomaculum* (Campbell and Postgate, 1965). The first isolate of this genus, a moderate thermophile, was originally described as *Clostridium nigrificans* (Werkman and Weaver, 1927), later as *Sporovibrio desulfuricans* (Starkey, 1938), and finally was named *Desulfotomaculum nigrificans* (Campbell and Postgate, 1965). Subsequently, some other moderately thermophilic and some mesophilic *Desulfotomaculum* species were isolated (Table 1). The genus exhibits a great nutritional versatility, comparable to that of nonsporeforming sulfate reducers (see Chapter 24). H_2, alcohols, fatty acids, other aliphatic monocarboxylic or dicarboxylic acids, alanine, hexoses, or phenyl-substituted organic acids may be used as electron donors for dissimilatory sulfate reduction (Table 1). Utilization of polysaccharides or polypeptides has not been reported.

Figures 1 and 2 show morphological features of a few *Desulfotomaculum* species. They are true Gram-positive bacteria and thus phylogenetically separate from other sulfate-reducing eubacteria, as is evident from their cell wall structure (Nazina and Pivovarova, 1979; Sleytr et al., 1969; Fig. 2B) and from 16S rRNA analyses (Devereux et al., 1989; Fowler et al., 1986). *Desulfotomaculum* species cluster with the branch of Gram-positive bacteria with DNA of low GC content (Fig. 3). *Desulfotomaculum* species may be regarded as clostridia-like bacteria which have the additional capacity for dissimilatory sulfate reduction.

Habitats

Sporeforming sulfate reducers thrive essentially in the same habitats as nonsporeforming types. However, *Desulfotomaculum* species have been isolated less frequently than Gram-negative sulfate reducers from habitats that are permanently or usually anoxic. In these environments, sporeforming species are apparently less competitive than nonsporeforming ones. In contrast, if conditions are prevailing that are selective for bacteria with spores, *Desulfotomaculum* species appear to be the predominant sulfate reducers in the particular environment. In contrast to nonsporeforming sulfate reducers, because of its spores, *Desulfotomaculum* is able to survive dryness and oxic conditions for many months or even years. If the conditions turn again anoxic, *Desulfotomaculum* species are the first sulfate reducers to develop. Such a selection for *Desulfotomaculum* has been observed in rice paddies where oxic and anoxic conditions alternate due to seasonal flooding. Anoxic samples collected during the flooding period from the rhizosphere of rice contained *Desulfotomaculum* as the predominant sulfate reducer (V. A. Jacq, personal communication). Also, in some other cases, the source of isolation reflected the survival of *Desulfotomaculum* species as spores. *D. nigrificans* was found in canned food (Werkman and Weaver, 1927), and *D. orientis* (Adams and Postgate, 1959) and *D. sapomandens* (Cord-Ruwisch and Garcia, 1985) originate from soil. Actually, spores of lactate- or fatty acid-oxidizing *Desulfotomaculum* species are regularly detected in oxic humid or dry soil, even if it has never been anoxic (F. Widdel, unpublished observations). Spores are probably spread by wind or animals and remain viable for years.

In contrast to sulfate-reducing bacteria of the gamma subdivision, the genus *Desulfotomaculum* contains a number of moderately thermophilic species with temperature optima of 54 to 65°C (Table 1). A *D. nigrificans* strain was isolated from hot-oil-field water (Nazina and Rozanova, 1978). *D. geothermicum* and *D. kuznetsovii* were obtained from geothermal ground water, and *D. thermoacetoxidans* originated from a thermophilic biogas fermenter. Hence, sulfate reduction in habitats with temperatures between 50 and 65°C can mainly be due to *Desulfotamaculum* species. However, at temperatures above 60 to 65°C, *Thermodesulfobacterium* species may

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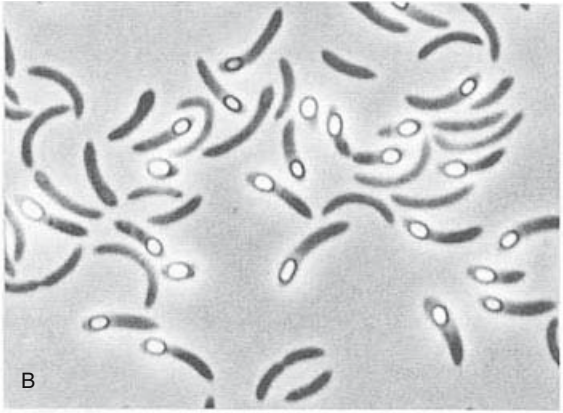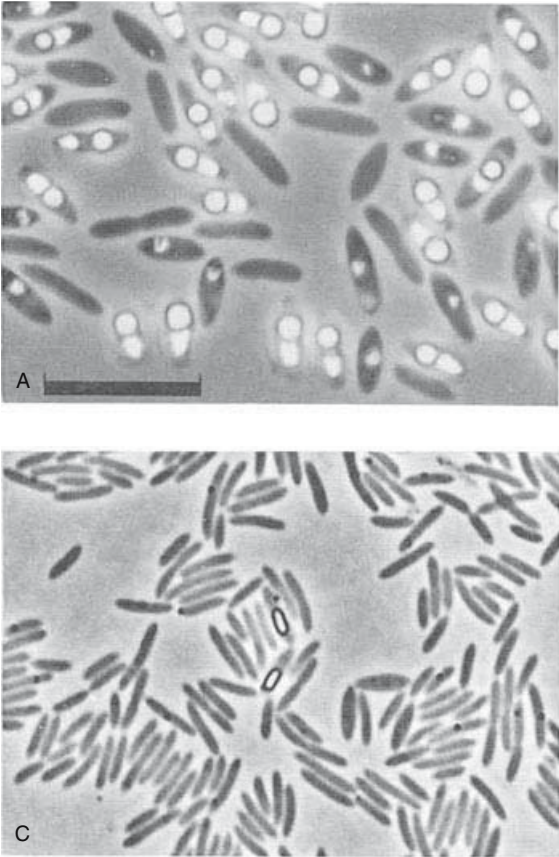
Fig. 1. Phase contrast photomicrographs of viable cells of sporeforming sulfate-reducing bacteria. Bar = 10 μm. (A) *D. acetoxidans* with spores, from a colony grown in agar with acetate. (B) *D. orientis* with spores, from a culture grown under sulfate limitation with H₂. (Courtesy of H. Cypionka.) (C) *D. ruminis* with two spores; *D. nigrificans* has the same size and morphology.
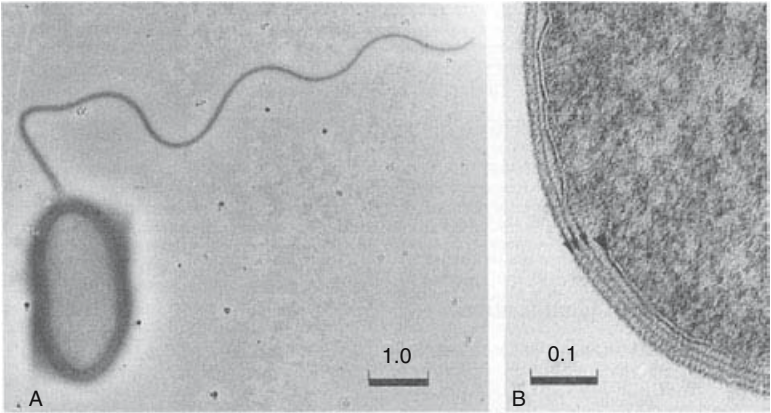
Fig. 2. Electron micrographs of spore-forming sulfate-reducing bacteria. (A) Negatively stained cell of *D. acetoxidans* showing the polar flagellum. (Courtesy of R. Lurz.) (B) Ultrathin section of a *D. nigrificans* strain from an oil field. The cytoplasmic membrane is indicated by the large arrowhead, the murein wall by the small arrows. (Courtesy of T. N. Nazina and T. A. Pivovarova.)
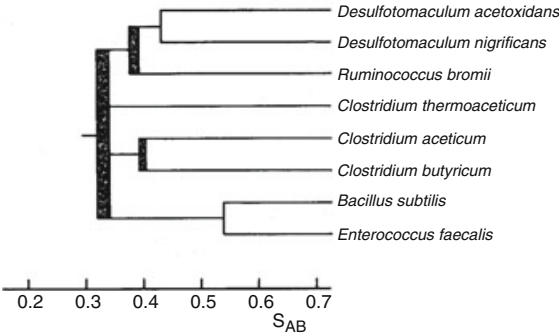
Fig. 3. Relationship of *D. nigrificans* and *D. acetoxidans* to each other and to other bacteria, revealed by 16S rRNA oligonucleotide cataloging. Dendrogram based on the data of Fowler et al. (1986), Stackebrandt et al. (1987), and E. Stackebrandt (personal communication). Relationships of two other *Desulfotomaculum* species are shown in Fig. 6 of Gram-Negative Mesophilic Sulfate-Reducing Bacteria in the second edition.

Table 1. Properties of *Desulfotomaculum* species.

Species	Morphology	Width (μm)	Length (μm)	Motility[a]	GC content of DNA (mol%)	Major menaquinone[b]	Temperature optimum (°C)	Oxidation of organic substrates
Desulfotomaculum								
acetoxidans	Straight or curved rod	1–1.5	3.5–9	+ (sp)	38	MK-7	34–36	Complete
antarcticum	Rod	1–1.2	4–6	+ (pe)	ND	ND	20–30	Incomplete
geothermicum	Rod	0.5–0.8	2.3–2.5	+	50	ND	54	Complete
guttoideum	Rod, drop-shaped	1	2.3	+ (pe)	48	ND	31	Incomplete
kuznetsovii	Rod	1–1.4	3.5–5	+ (pe)	49	ND	60–65	Complete
nigrificans[c]	Rod	0.5–0.7	2–4	+ (pe)	45	MK-7	55	Incomplete
orientis	Straight or curved rod	0.7–1	3–5	+ (pe)	45	MK-7	37	Incomplete
ruminis	Rod	0.5–0.7	2–4	+ (pe)	49	MK-7	37	Incomplete
sapomandens	Rod	1.2–2	5–7	+	48	ND	38	Complete
thermoacetoxidans	Straight or curved rod	0.7	2–5	+	50	ND	55–60	Complete

[a]Flagellation pattern indicated in parentheses: pe, peritrichous; sp, single and polar.
[b]See Collins and Widdel (1986); ND, not determined or not reported.
[c]Type species.
[d]Symbols: +, utilized; +*, autotrophic growth; (+), poorly utilized; –, not utilized; ND, not determined.
[e]Not completely listed; for further substrates, see references.
[f]Symbols: bi, biotin; pa, *p*-aminobenzoate.
[g]Type strain does not require NaCl. Another strain requires 5 g NaCl per liter for optimum growth (Nazina and Rozanova, 1978).

also play a role; however, they cannot oxidize acetate or fatty acids that are utilized by part of the thermophilic *Desulfotomaculum* species. If dissimilatory sulfate reduction occurs at temperatures of around 80°C or higher, this is probably due to archaebacterial sulfate reducers (see The Genus *Archaeoglobus* in Volume 3).

Most *Desulfotomaculum* species that have been described grow best at low salt concentrations and thus seem to be dwellers mainly of freshwater habitats or other aqueous environments with relatively low salt concentrations. However, *D. geothermicum* isolated from saline geothermal ground water, grew optimally with 20 to 30 g NaCl per liter (Daumas et al., 1988). A *D. nigrificans* strain from hot-oil-field water

required 5 g NaCl per liter for optimum growth (Nazina and Pivovarova, 1979). Two other, unnamed types that were probably adapted to a saline environment were a benzoate-utilizing strain from an oil field (Cord-Ruwisch et al., 1986) and an acetate-utilizing strain from marine sediment (Keith et al., 1982).

D. ruminis has been isolated from sheep rumen (Coleman, 1960). However, with 10^2 cells/ml, this type was present in far lower numbers than *Desulfovibrio*, which exhibited an abundancy of 10^7 cells/ml (Howard and Hungate, 1976). *Desulfotomaculum acetoxidans* could be readily and repeatedly enriched from animal manure with butyrate as energy source (Widdel and Pfennig, 1977, 1981; F. Widdel, unpublished observations).

H₂	Formate	Acetate	Fatty acids: C atoms	Ethanol	Lactate	Fumarate	Malate	Benzoate	Others[e]	Growth factor requirement[f]	NaCl requirement (g/l)	References
									Electron donors for sulfate reduction[d]			
−	(+)	+	4–5	+	−	−	−	−	Butanol	bi	—	Widdel and Pfennig, 1977, 1981
ND	−	−	ND	ND	+	ND	ND	ND	Glucose	Unknown	—	Iizuka et al., 1969
+*	+	−	3–18	+	+	ND	ND	−	Fructose	Unknown	25	Daumas et al., 1988
+	−	−	ND	−	+	ND	−	ND	—	Unknown	—	Gogotova and Vainshtein, 1983
+*	+	+	3–16	+	+	+	+	−	Methanol, propanol, butanol	Unknown	—	Nazina et al., 1988
+	+	−	−	+	+	−	−	−	Fructose, alanine	Unknown	—	Campbell and Postgate, 1965; Klemps et al., 1985
+*	+	−	−	+	+	−	−	−	Methanol, 3,4,5-trimethoxybenzoate	Unknown	—[g]	Campbell and Postgate, 1965; Klemps et al., 1985
+	+	−	−	+	+	−	−	−	Alanine	bi, pa	—	Campbell and Postgate, 1965; Klemps et al., 1985
ND	+	(+)	4–18	+	−	+	+	+	Isobutyrate, 3-methylbutyrate, phenylacetate	Unknown	—	Cord-Ruwisch and Garcia, 1985
+*	+	+	3–5	−	+	ND	+	−	Propanol, butanol, alanine	Unknown	—	Min and Zinder, 1990

Cultivation Techniques and Media

For cultivation of *Desulfotomaculum* species, the same vessels, equipment, and anoxic techniques as described in detail for Gram-negative sulfate reducers (see Chapter 183) may be used. However, the choice of the reductant can be critical and needs special attention.

Use of Reductants

Desulfotomaculum nigrificans, *D. orientis*, *D. ruminis*, and some other incompletely oxidizing species that were originally isolated on the lactate medium with ascorbate and thioglycollate as reductants (see Gram-Negative Mesophilic Sulfate-Reducing Bacteria in the second edition.) show very poor growth in media reduced with commercial sodium sulfide (Klemps et al., 1985; F. Widdel, unpublished observations). The inhibition by Na₂S is probably not due to sulfide itself, since the added concentration (around 1.5 mM) is far below the concentration that is usually inhibitory to *Desulfotomaculum* species (7 to 10 mM; e.g. Coleman, 1960; Cord-Ruwisch and Garcia, 1985). It is more likely that certain impurities in the commercial sodium sulfide are inhibitory.

The indicated *Desulfotomaculum* species are therefore grown in the lactate medium for *Desulfovibrio*, which is prereduced with ascorbate and thioglycollate. Alternatively, the defined

multipurpose medium (Gram-Negative Meso-philic Sulfate-Reducing Bacteria in the second edition) may be used if ascorbate and thioglycol-late are added instead of Na$_2$S. Bicarbonate/CO$_2$, various vitamins, and trace elements present in the multipurpose medium may be required by several species.

Dithionite has also been used as sole reductant for cultivation of *Desulfotomaculum* species (e.g., Cypionka and Pfennig, 1986). Even though some *Desulfotomaculum* species may be less sensitive toward this reducing agent than other anaerobes, dithionite is probably toxic if added at the same concentration as other reductants. It is therefore added immediately before inocula-tion and only in the smallest amount sufficient to reduce the medium. To assess the redox status, an indicator such as resazurine is added (from a 0.1% (wt/vol) solution of resazurine (sodium salt), 1 ml is added per liter of medium). Sodium dithionite is added from a fresh solution prepared under N$_2$ or as dry powder (Gram-Negative Mesophilic Sulfate-Reducing Bacteria in the sec-ond edition) until resazurine is decolorized. Then, the culture vessel is inoculated and imme-diately sealed, either with a screw cap (in case of completely filled culture vessels) or with a stop-per (in case of vessels with an anoxic head space).

Desulfotomaculum ruminis was isolated in sulfite agar and shown to tolerate sulfite up to a concentration of several mM. Even though the use of Na$_2$SO$_3$ as reductant is not very common, it should be considered for new media for *Desulfotomaculum*.

Desulfotomaculum guttoideum and species using fatty acids have been isolated in Na$_2$S-reduced media and can therefore be grown with this reductant. However, growth in the afore-mentioned media with other reductants may be also attempted. If Na$_2$S is used, dithionite (15 to 30 mg/l) as an additional reductant often signifi-cantly favors initial growth (Widdel and Pfennig, 1977, 1981).

Other Variations of Standard Media

A nonchelated trace element solution such as the solution 1 described in Chapter 183 has been used for cultivation of many *Desulfotomaculum* species (Cord-Ruwisch and Garcia, 1985; Dau-mas et al., 1988; Gogotova and Vainshtein, 1983; Widdel and Pfennig, 1977, 1981). Furthermore, trace elements chelated with EDTA (ethylenedi-aminetetracetic acid, neutralized) or NTA (nitrilotriacetic acid, neutralized) have been used. The EDTA-containing solution (Nazina et al., 1988) had essentially the composition of solu-tion 2. In case of NTA, the composition was as in solution 1, but with 12.8 g NTA (Cypionka and

Pfennig, 1986), or as in solution 3, but with only 4.5 g NTA (Min and Zinder, 1990).

An excellent organic substrate for rapid growth of incompletely oxidizing, lactate-utilizing *Desulfotomaculum* species is pyruvate. Only half as much H$_2$S is formed per mol of pyruvate oxidized to acetate, as per mol of lac-tate oxidized. Therefore, high cell densities can be reached on pyruvate before H$_2$S becomes inhibitory. A stock solution of 2 M pyruvate is prepared from the sodium salt (22 g in 100 ml) or from the cheaper free acid. In the latter case, 14.4 ml (17.6 g) pyruvic acid is added to 40 ml distilled H$_2$O. Then, the solution is neutralized by slow addition of 4 M NaOH and finally of 1 M NaOH under stirring in an ice bath. H$_2$O is added to a final volume of 100 ml. The sodium pyruvate solution is filter-sterilized and stored in the dark at 4°C. Per liter of medium, 15 to 20 ml pyruvate solution are added.

Desulfotomaculum nigrificans does not grow well in defined medium; this species is signifi-cantly stimulated by yeast extract that is present in the lactate medium (Gram-Negative Meso-philic Sulfate-Reducing Bacteria in the second edition). If the multipurpose medium is used, yeast extract is added at the same concentration (1 g/l). A somewhat weaker but distinct stimula-tion in the multipurpose medium was also achieved with a mixture of 60 mg alanine, 90 mg asparagine, 15 mg cysteine, and 85 g threonine instead of yeast extract per liter of medium, and a threefold increased FeSO$_4$ concentration (F. Widdel, unpublished observations). Growth of other incompletely oxidizing *Desulfotomaculum* species is also promoted by yeast extract, but the effect is less pronounced that in the case of *D. nigrificans*. Yeast extract has no effect on the growth of *D. acetoxidans*.

Enrichment, Isolation, and Maintenance

Enrichment

The formation of heat- and drought-resistant spores in *Desulfotomaculum* species allows selective enrichment in the presence of other sulfate reducers. To eliminate nonsporeformers, samples from sediments or other anoxic habitats are pasteurized at 80°C for 10 to 20 min. To guarantee uniform heating, the sample volume is kept small (<10 ml). Pasteurization may be car-ried out in test tubes or bottles incubated in a thermostated water bath. Exclusion of air is not necessary.

Selective enrichment of *Desulfotomaculum* species is also possible from samples of oxic surface soil (see "Habitats," this chapter). Since

nonsporeforming sulfate reducers are usually absent from such soil, *Desulfotomaculum* species are selectively enriched even from nonpasteurized soil samples.

In enrichments with nonpasteurized samples from organic-rich, anoxic habitats (sediments, sludge), *Desulfotomaculum* species are usually outcompeted by nonsporeforming sulfate reducers. An exception seem to be enrichments under moderately thermophilic conditions (50 to 60°C), which often yield *Desulfotomaculum* species even without preceding pasteurization. This is probably due to the lack of nonsporeforming sulfate reducers adapted to this temperature range in these kinds of habitats (see "Habitats," this chapter).

Besides temperature, selective factors for enrichment of various types of *Desulfotomaculum* include the electron donor and the salt concentration. *D. ruminis*, *D. orientis*, and nutritionally similar incomplete oxidizers may be enriched with lactate. Enrichment with an H_2/CO_2 mixture in the presence or absence of some acetate (1 mM) has not been tested but may be also successful. There is, however, no one method that guarantees selection of a particular species. Only *Desulfotomaculum acetoxidans* could be regularly enriched from cow or pig manure with acetate, butyrate, or isobutyrate as electron donors, even without preceding pasteurization (Widdel and Pfennig, 1977, 1981; F. Widdel, unpublished observations).

Various types of sporeforming sulfate reducers may be also obtained by serial dilution of soil or pasteurized mud samples in agar deeps (see below and Gram-Negative Mesophilic Sulfate-Reducing Bacteria in the second edition).

Isolation

Desulfotomaculum species are isolated via serial dilutions in agar deeps or using one of the other procedures described in Chapter 183.

Maintenance

Like many sporeforming bacteria, vegetative cells of *Desulfotomaculum* species die off very soon after growth has ceased, especially if kept at optimal temperature. Many species that grow well lose their viability within 2 to 4 days. Since spores are usually not formed in cultures growing under optimal conditions, strains are easily lost upon prolonged incubation. The rate of cell death may be reduced by growth at suboptimal temperature (e.g., 20°C). However, the best method for preservation of *Desulfotomaculum* cultures is in the sporulated state. Spore formation is often induced in colonies in agar deeps. Strains to be maintained are therefore diluted in agar as described for isolation of pure cultures (see

Gram-Negative Mesophilic Sulfate-Reducing Bacteria in the second edition). Agar tubes with fully grown colonies are incubated for several more weeks to allow completion of sporulation.

Formation of some spores in cultures of *Desulfomaculum acetoxidans* may also occur during slow growth on acetate at suboptimal temperature (20°C). This species never forms spores on butyrate, the preferred substrate for rapid germination of spores and for growth (Widdel and Pfennig, 1981).

Desulfotomaculum orientis forms spores if grown in sulfate-limited cultures with H_2 as electron donor (Cypionka and Pfennig, 1986; Fig. 1B).

Agar tubes with spore-containing colonies are refrigerated. For revival, colonies are picked and transferred to fresh liquid medium. Sporulated liquid cultures may be freeze-dried with skim milk or with the precipitated ferrous sulfate formed in the iron-rich lactate medium B (Gram-Negative Mesophilic Sulfate-Reducing Bacteria in the second edition) and kept at –20°C.

Taxonomy and Identification

The genealogical affiliation of four *Desulfotomaculum* species with the branch of Gram-positive bacteria with low GC content has been demonstrated by oligonucleotide cataloging (Fowler et al., 1985; Fig. 3) and by the sequencing (Devereux et al., 1989) of 16S rRNA. Comparative genealogical studies on *Desulfotomaculum* species on a larger scale are lacking thus far. Hence, it is not known whether there is a coincidence of physiological and phylogenetic groupings within the genus. The physiological variations in the genus (Table 1) and the relatively low degree of relatedness even between the nutritionally similar *D. orientis* and *D. ruminis* (Devereux et al., 1989; Chapter 183, Fig. 6) indicates that the genus is less coherent than any other genus of Gram-negative sulfate reducers. However, as long as the genus *Clostridium*, with its multiple physiological types, is not subdivided, there is no reason to do so with the genus *Desulfotomaculum*. For the present, sporeforming sulfate reducers can be unequivocally identified as members of the genus *Desulfotomaculum*. A brief description of characteristics of the genus is given in the following. Properties of particular species are listed in Table 1. Figures 1 and 2 show morphological features of a few species.

Genus *Desulfotomaculum*

The common property of *Desulfotomaculum* species is dissimilatory sulfate reduction to sulfide and the formation of endospores. The location of spherical or oval spores may be central, subterminal, or terminal. In some species, gas vacuoles

are formed simultaneously with spores (Daumas et al., 1988; Widdel and Pfennig, 1977, 1981; Fig. 1A). Spores are resistant to heat (80°C, 10 to 20 min), dryness, and oxic conditions. *Desulfotomaculum* species grow with H_2, alcohols, a few sugars, or simple organic acids including phenyl-substituted ones that serve as electron donors (Table 1). Some species oxidize organic electron donors incompletely to acetate. Species utilizing fatty acids or benzoate are capable of complete oxidation, even though these organic substrates may be partially converted to acetate (Daumas et al., 1988; Widdel and Pfennig, 1981). H_2-utilizing species may be capable of auto-trophic growth (Brysch et al., 1987; Daumas et al., 1988; Klemps et al., 1985; Min and Zinder, 1990; Nazina et al., 1988). In the absence of sulfate, some species grow by acid fermentation of glucose, fructose, or pyruvate. Certain *Desulfomaculum* species resemble homoacetogenic bacteria (see Chapter 21) in their ability to convert methanol, methoxyl groups of 3,4,5-trimethoxybenzoate, formate, and even H_2 and CO_2 to acetate (K. Hanselmann, personal communication; Klemps et al., 1985; Min and Zinder, 1990). This homoacetogenesis may allow weak growth in the absence of sulfate. Sulfite or thiosulfate can replace sulfate as electron acceptor in many species. *Desulfotomaculum* species are more sensitive toward the sulfide produced than most Gram-negative sulfate reducers. Whereas the latter often grow up to H_2S concentrations of around 20 mM, *Desulfotomaculum* species are inhibited by 7 to 10 mM H_2S. Desulfoviridin has never been found in the genus *Desulfotomaculum*; however, the sulfite reductase P582 is present, as shown by the characteristic carbon monoxide difference spectrum of reduced cell extracts (Trudinger, 1970; Widdel and Pfennig, 1977). It had originally been assumed that *Desulfotomaculum* species contain only cytochromes of the *b*-type, but *c*-type cytochromes have been detected as well (e.g., Daumas et al., 1988; Gogotova and Vainshtein, 1983; Jones, 1972).

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CHAPTER 1.2.26

The Anaerobic Gram-Positive Cocci

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Introduction

The anaerobic Gram-positive cocci (also known as peptococci and peptostreptococci) discussed in this chapter are limited to firmicutes with low mol% G+C. They are *Peptococcus*, *Peptostreptococcus*, *Anaerococcus*, *Peptoniphilus*, *Gallicola*, *Finegoldia*, *Micromonas*, *Ruminococcus*, *Coprococcus* and *Sarcina*. All except *Anaerococcus*, *Peptoniphilus*, *Gallicola*, *Finegoldia* and *Micromonas* belong to the classical family Peptococaceae (Rogosa, 1974). During the last 10 years, many members of the old genus *Peptostreptococcus* were transferred to many other genera. Most anaerobic Gram-positive cocci belong to the normal flora of the human gastrointestinal tract, vagina and oral cavity and often are found in human clinical specimens (Brook and Frazier, 1990; Ezaki et al., 1992). (Most human clinical isolates were identified as peptostreptococci.) Phylogenetically, anaerobic Gram-positive cocci belong to the phylum *Clostridium*. Indeed, based on 16S rRNA sequences, all species of the genus Peptostreptococcus (Li et al., 1994; Collins et al., 1994) were in different groups in this phylum. *Peptococcus niger* was close to the clostridial cluster III (Collins et al., 1994). *Sarcina ventriculi* was close to the clostridial cluster I (Li et al., 1994; Willems and Collins, 1994), and the ruminococci and coprococci were within clostridial cluster XIV (Willems and Collins, 1995; Rainey and Janssen, 1995).

Members of the genus *Peptostreptococcus* also were clustered into three groups within the phylum Clostridium: *P. anaerobius* within clostridial cluster XI, "*P. heliotrinreducens*," recently transferred to a new genus *Slackia* (a high mol% G+C firmicute; Wade et al., 1999), and the remaining species classified with clostridial cluster XIII.

Accumulated chemotaxonomic data also proved that peptostreptococci were in different groups. Old peptostreptococci have several peptidoglycan structures and their structures differ from that of the type species, *P. anaerobius* (Li et al., 1992; Murdoch et al., 1997). Using these data, peptostreptococci were recently reclassi-fied into five genera: *Anaerococcus*, *Peptoniphilus*, *Finegoldia*, *Micromonas* and *Gallicola*.

Habitat

Anaerobic cocci are part of the normal flora of the human oral cavity, alimentary tract, skin and vagina (Moore and Holdeman, 1974; Holdeman et al., 1986; Tanner and Stillmann, 1993). Among them, Micromonas micros and *Finegoldia magna* often are isolated from sites of oral infection and otorrhea (Shah and Gharbia, 1995). Sarcinae are isolated from soil but also from the digestive tract of humans and animals (Canale-Parola, 1970; Canale-Parola, 1986). The anaerococci, peptoniphili, peptostreptococci, finegoldiae and micromonades are found in the mouth and upper respiratory tract and in the lower small intestine, colon and vagina (Finegold and George, 1989; Murdoch, 1998; Tanner and Stillmann, 1993). *Ruminococcus obeum* and *R. bromii* are often isolated from the large intestine of humans (Moore et al., 1974). Anaerococci, peptoniphili and finegoldiae are frequently found in the vagina (Marui, 1981; Finegold, 1995). In particular, the species *Finegoldia magna*, *Peptoniphilus asaccharolyticus*, *Anaerococcus prevotii*, *A. hydrogenalis*, *A. tetradius*, *A. vaginalis*, *Ps. anaerobius*, *Peptococcus niger* and *Finegoldia magna* are common isolates from human skin (Wilkins and Jimenez-Ulate, 1975; Finegold, 1995). *Finegoldia magna*, *A. prevotii* and *P. asaccharolyticus* are also commonly in human and animal stools.

Anaerobic cocci have been known to be associated with sepsis, septic thrombophlebitis, puerperal fever, sinusitis and otitis media (Bartlett, 1993; Finegold, 1995). Early in the 20th century, anaerobic cocci or anaerobic streptococci were not referred to as true anaerobes. Capnophilic streptococci (anaerobic strains) were once placed in the genus *Peptococcus* and were later transferred to the genus *Streptococcus* (Holdeman and Moore, 1974). These include *S. intermedius*, *S. constellatus* and *S. anginosus*, and "*S. parvulus*" (now classified as *Atopobium parvulum*). *Staphylococcus saccharolyticus* (another

anaerobe), isolated from the skin (Evans et al., 1978), also was misidentified as true anaerobic cocci. Major Gram-positive anaerobic cocci isolated from various clinical specimens include members of the genus *Anaerococcus*, *Peptoniphilus*, *Finegoldia*, *Micromonas* and *Peptostreptococcus*, but not generally ruminococci, coprococci or sarcinae.

Isolation

Media for anaerobes should be stored under anaerobic conditions. Agar plates for the isolation of anaerobic cocci should be kept in anaerobic jars or chambers. The plates for ruminococci, coprococci and sarcinae should be kept under more reduced conditions. These organisms require carbohydrate to grow. Ruminococci and coprococci only grow in roll tubes or anaerobic chambers (such as anaerobic glove boxes) filled with a gas mixture of 80% nitrogen (N_2), 10% hydrogen (H_2) and 10% carbon dioxide (CO_2). Commercial gas is often contaminated with trace amounts of (O_2) oxygen; therefore, a palladium (Pd) catalyst should be placed in an anaerobic chamber to remove O_2. Because these chambers are O_2-free environments, they are useful, as well, for the isolation of O_2-sensitive bacteria. A rather inexpensive alternative to the anaerobic chamber method, the roll tube method (Hungate, 1966) was originally developed to isolate rumen bacteria and later modified by Holdeman et al. (1977). Broth and solid media are placed in tubes with rubber stoppers to prevent O_2 exposure. The media (called "prereduced anaerobically sterilized" [PRAS]) should be prepared under an O_2-free gas that is passed through a cold catalyst or heated copper catalyst. These PRAS agar plates and broth media are available commercially. Another culture technique for small clinical laboratories is the anaerobic jar method. One jar can hold from 10 to 20 agar plates. The jar (commercially available from e.g., Oxoid, Merck, BBL, or Mitsumishi Gas) includes a gas-generation bag, an O_2 indicator and a catalyst. For a single plate culture, an anaerobic pack, which is supplied with O_2-absorbing reagents and a CO_2 generator, is also commercially available (e.g., from Oxoid, Mitsubishi Gas or Dia Iatoron).

Primary Isolation Media for *Anaerobic Cocci* Not Requiring Fermentable Carbohydrates

Anaerobic cocci have complex growth requirements, which may include vitamins, cofactors and amino acids. Tween 80 (at a final concentration of 0.02%) enhances the growth of these organisms but is not required for their isolation. These cocci metabolize peptones and amino acids; therefore, a complex medium, such as blood agar or chopped meat medium, is necessary for isolation. Basal media for blood agar plates include Brucella agar (BBL), Schaedler agar (BBL), Colombia agar (Difco), and brain heart infusion agar; all basal media must be supplemented with yeast extract, vitamin K, hemin and 5% sheep's blood. Brucella HK agar (Kyokuto, Japan) is premixed with vitamin K and hemin. For the isolation of anaerobic cocci, media without blood, such as chopped meat agar, modified GAM agar (Nissui, Japan), ABCM agar (Eiken, Japan), and Brucella HK semisolid agar, are also useful. For the selective isolation of the anaerobic cocci from contaminated specimens, a phenylethyl alcohol blood agar (Difco, BBL) plate is recommended.

Isolation Media for *Ruminococci*, *Coprococci* and *Sarcinae*

Ruminococci (Bryant, 1986) and coprococci (Holdeman and Moore, 1986) require fermentable carbohydrates and ammonia for growth. Selective media for the primary isolation of these organisms have not been developed, except for the isolation of cellulolytic organisms. Unless special nutrients are added, currently available commercial media do not support growth, although ruminococci and coprococci grow well on media containing 30–40% rumen fluid.

Rumen Fluid-Glucose-Cellobiose Agar (Holdeman et al., 1977)

Glucose	0.25 g
Cellobiose	0.25 g
Soluble starch	0.5 g
$(NH_4)2SO_4$	1.0 g
Rumen fluid	300.0 ml
Cysteine-HCl · H_2O	0.5 g
Resazurin solution (see below)	4.0 ml
Salts solution (see below)	500.0 ml
Hemin solution (see below)	10.0 ml
Agar	20.0 g
Distilled water	186.0 ml

Rumen fluid can be replaced with a volatile fatty acid (VFA) mixture, as in medium 10 (see below; Caldwell and Bryant, 1966).

Resazurin Solution

Twenty-five mg of resazurin is dissolved in 100 ml of distilled water.

Salts Solution

$CaCl_2$	0.2 g
$MgSO_4$	0.2 g
$KIHPO_4$	1.0 g
KH_2PO_4	1.0 g
$NaHCO_3$	10.0 g
NaCl	2.0 g
Distilled water	1,000.0 ml

Hemin Solution

Fifty mg of hemin in 1 ml of 1 N NaOH is diluted up to 100 ml with distilled water.

Medium 10 (Caldwell and Bryant, 1966)

Trypticase (BBL)	2.0 g
Yeast extract (Difco)	0.5 g
Hemin solution	2.0 ml
Glucose	0.5 g
Cellobiose	0.5 g
Soluble starch	0.5 g
Agar	18.0 g
Resazurin	1.0 ml
0.69% K_2HPO_4	37.5 ml
Mineral solution 1	37.5 ml
VFA mixture	3.1 ml
L-Cysteine	10.0 ml
Na_2CO_3	50.0 ml
Ascorbic acid	2.0 ml
Distilled water	857.0 ml

Details of the preparation of the original Medium 10 are given in Holdeman et al. (1977).

Mineral Solution l

KH_2PO_4	0.6	g
$(NH_4)2SO_4$	0.6	g
NaCl	1.2	g
$MgSO_4 \cdot 7H_2O$	0.25	g
$CaCl_2 \cdot 2H_2O$	0.16	g

VFA Mixture

Acetic acid	17 ml
Propionic acid	6 ml
N-Butyric acid	4 ml
N-Valeric acid	1 ml
Isovaleric acid	1 ml
Isobutyric acid	1 ml
DL-a-Methylbutyric acid	1 ml

Selective isolation of cellulolytic ruminococci from rumen was established by Hungate (1966). Presumptive identification of cellulose digesters grown on rumen fluid-amorphous cellulose-agar plates is based on the appearance of typical colonies surrounded with a clear zone resulting from cellulose digestion.

Primary Isolation of Sarcinae

Selective media for the primary isolation of sarcinae are based on two distinct characteristics: 1) fermentable carbohydrates stimulate their growth and 2) they can grow at low pH (2.0–2.5). Sarcinae are often found in feces of humans when Bifidobacterium-selective medium, which contains tomato juice and maltose, is used.

Bifidibacterium Medium

Canned tomato juice	45.5 g
Maltose	10 g
Hemin solution	2 ml
Distilled water	600 ml

Adjust pH to 5–6.

A different medium is selective for sarcinae from soil.

Selective Enrichment Broth for the Isolation of Sarcinae from Soil (Canale-Parola, 1970)

Maltose	2.0 g
Malt extract broth (BBL)	5.0 g
Tap water	1,000 ml

Adjust to pH 2.2 with H_2SO_4.

Preservation of Cultures

Anaerobic cocci grow well in PRAS-chopped meat broth, GAM semisolid and Brucella HK semisolid medium.

PRAS-Chopped Meat Broth (Holdeman et al., 1977)

Ground beef (fat-free)	500 g
Distilled water	1,000 ml
1 N NaOH	25 ml

Boil the above mixture, then cool to room temperature. Skim off the fat and filter the broth. Add distilled water to restore to the original volume and add 30 g of trypticase, 5 g of yeast extract, 5 g of potassium phosphate, and 4 ml of 0.025% resazurin solution. Boil under O_2-free N_2 gas and add 0.5 g of cysteine, 10 ml of 0.05% hemin solution, and 1 µl of vitamin Kl solution. Adjust pH to 7.2 ± 0.2. Prepare the test tubes containing the meat particles (use one part meat particles to four to five parts fluid). Dispense 3–5 ml of the broth into the tubes filled with O_2-free CO_2 gas; seal the tubes with rubber stoppers, then autoclave.

Alternatively, because preparation of PRAS-chopped meat broth is time consuming, we often prepare semisolid GAM medium or modified semisolid Brucella medium (Kyokuto Seiyaku, Tokyo, Japan) in ordinary test tubes. *Anaerococci*, *Peptoniphili*, *Finegoldiae* and *Micromonades* grow well in this semisolid medium.

Modified Brucella Semisolid Medium for Maintenance of *Peptococci*, *Peptostreptococci*, *Finegoldiae* and *Micromonades*

Casein peptone	10.0 g
Beef peptone	10.0 g
Yeast extract	5.0 g
Soy peptone	4.0 g
Glucose	1.0 g
Sodium sulfite	0.1 g
Arginine	1.0 g
Vitamin K	1.0 µl
Hemin	0.01 g
Sodium pyruvate	1.0 g
Sodium fumarate	0.2 g
K_2HPO_4	0.4 g
KH_2PO_4	0.8 g
NaCl	2.5 g
Sodium thioglycolate	0.3 g
Soluble starch	5.0 g
Cysteine HCl	0.3 g
Agar	1.5 g
Distilled water	1,000 ml

These semisolid media can be used for at least one week after preparation. Indicators, such as resazurin, of oxygenated areas can be added to the semisolid media. For the maintenance of ruminococci, coprococci and sarcinae, semisolid PRAS-chopped meat medium supplemented with various carbohydrates (Holdeman et al., 1977) is recommended. The rubber stopper of the test tube is opened without flushing the inside of the tube with gas. After inoculation of the organisms with a Pasteur pipette, the test tube should be sealed with a rubber stopper. Most ruminococci and coprococci may grow in this way, but they grow better if the tube is filled with a gas mixture (N_2, 80%; CO_2, 10%; H_2, 10%). Brucella HK and GAM semisolid media support the growth of most anaerobic cocci. *Peptococcus niger* grows poorly in these commercial media, but the addition of pyruvic acid will stimulate the growth. *Ruminococci* and *coprococci* also grow poorly in Brucella HK and GAM, and so they are maintained in PRAS-chopped meat broth supplemented with fermentable carbohydrate (glucose, cellobiose or maltose at a concentration less than 0.2%). Sarcinae will die within one week in acidic enrichment broth. These anaerobic cocci are viable for at least several years at −80°C or in liquid nitrogen.

Basal Media for Identification

Pre-reduced peptone-yeast extract (PY) medium (Holdeman et al., 1977) is used for the characterization of anaerobic cocci. Most anaerobic cocci grow poorly in this medium. Thus a heavy inoculum of a young culture (5% of broth culture) is critical in this procedure. Commercial pre-reduced PY medium (Scot II) based on the description by Holdeman et al. (1977) is available.

Basal Prereduced Peptone-Yeast Extract (PY)
Medium (Holdeman et al., 1977)

Trypticase	5.0 g
Peptone	5.0 g
Yeast extract	10.0 g
Vitamin K1	1.0 µl
Hemin	5.0 g
Cysteine HCl	0.5 g
Salts solution	40.0 ml
Distilled water	960.0 ml

Identification

Phylogenetic Position of Anaerobic Gram-positive Cocci

Members of the family *Peptococaceae* (Rogosa, 1974) previously were divided into five phylogenetic groups in the phylum *Clostridium*. *Clostridia*, viewed as mixtures of phylogeneti-cally different organisms, were assigned to 19 phylogenetic groups (Collins et al., 1994). In this study, anaerobic cocci and many species of genus *Eubacterium* were found to be in the phylum *Clostridium*. *Peptococcus niger* was included in clostridial cluster III (Collins et al., 1994). *Sarcina ventriculi* was found to cluster closely with clostridial cluster I (Li et al., 1994; Willems and Collins, 1994), and ruminococci and coprococci with clostridial cluster XIV (Willems and Collins, 1995; Rainey and Janssen, 1995). Members of the genus *Peptostreptococcus* were in three different clostridial clusters. For example, *P. anaerobius* was in clostridial cluster XI. "*P. heliotrinreducens*" was recently transferred to a new genus *Slackia* (Wade et al., 1999). Chemotaxonomic data also proved that peptostreptococci grouped differently. Peptostreptococci have several different peptidoglycan structures, all of them different from that of the type species, *P. anaerobius* (Li et al., 1992; Murdoch et al., 1997). Taken together, the evidence suggested that five genera—*Anaerococcus*, *Peptoniphilus*, *Gallicola*, *Finegoldia* and *Micromonas*—be proposed (Murdoch and Shah, 1999; Ezaki et al., 2000). Thus, only the type species Peptostreptococcus anaerobius remained in the genus *Peptostreptococcus*. Their biochemical characteristics at genus level are shown in Fig. 1.

In the early 1970s, Rogosa (1971) proposed the family Peptococcaceae to which the genera *Peptococcus*, *Peptostreptococcus*, *Ruminococcus* and *Sarcina* were assigned. Later, Holdeman and Moore (1974) added the new genus *Coprococcus*. However, phylogenetic analysis of 16S rRNA of members of this family revealed that they were scattered in the phyla of Firmicutes with low mol% G+C (Fig. 2). Type species of the genus *Peptostreptococcus*, *P. anaerobius* was independent from other members of the genus *Peptostreptococcus* and clustered with clostridial cluster IX (Collins, 1994). Asaccharolytic and butyrate-positive group 1 peptostreptococci were now reclassified as genus *Peptoniphilus*. Weakly saccharolytic and butyrate-producing group 2 peptostreptococci were reclassified as genus *Anaerococcus*. Three species of group 3 peptostreptococci that carried different peptidoglycan structures were assigned to genera *Finegoldia*, *Micromonas* (Murdoch and Shah, 1999) and Gallicola (Ezaki et al., 2000). Their phylogenetic relationship is shown in Fig. 2.

Members of the genus *Ruminococcus* were clearly divided into two groups. Group 1 contains the type species, *R. flavefaciens*. Group 2 ruminococci and coprococci were grouped into clostridial cluster XIVa (Fig. 2). Packet-forming cocci, the sarcinae, were found to be close to *Clostridium perfringens*. Differential characteristics of these groups at genus level are listed in Table 1.

Fig. 1. Phylogenetic position of anaerobic cocci among firmicutes with low mol% G+C.
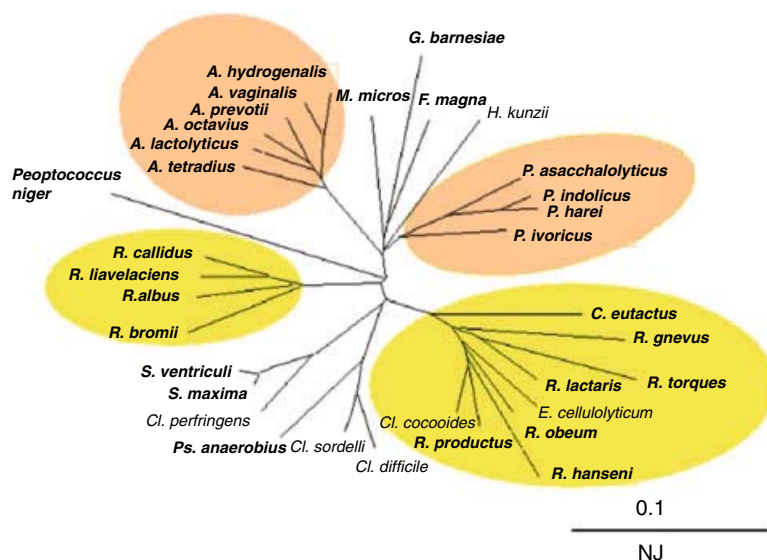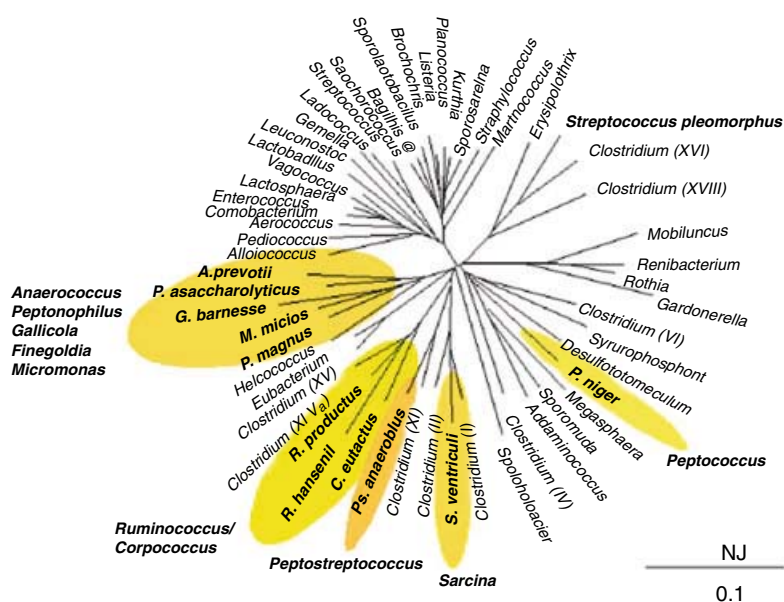
Fig. 2. Phylogeny of Gram-positive anaerobic cocci.

Identification

Biochemical Identification

The first step in the identification of anaerobic cocci is to distinguish anaerobic strains of streptococci and staphylococci from "true anaerobic cocci." *Streptococcus pleomorphus* is anaerobic and strains of *Gemella morbillorum* (Kilpper-Baelz and Schleifer, 1988), *S. anginosus*, *S. intermedius* and *S. constellatus* often grow only under strictly anaerobic conditions but most of them, after several transfers in the laboratory, eventually grow in an O$_2$-reduced environment or in a 5–10% CO$_2$ incubator. Anaerobic strains of streptococci and gemellae, unlike *Peptococcus*, *Peptostreptococcus*, *Ruminococcus*, *Coprococcus*, *Finegoldia*, *Micromonas*, *Gallicola* and *Sarcina*, solely produce lactic acid from PY medium containing glucose.

Colonies of *Staphylococcus saccharolyticus*, a species formerly assigned to the genus *Peptococcus*, often grow on anaerobic plates under reduced O$_2$ conditions (e.g., in a 5–10% CO$_2$ incubator; Evans et al., 1978) or more slowly in aerobic primary culture. *Staphylococcus aureus* subsp. anaerobius (Fuente et al., 1985) also grows under reduced O$_2$ conditions but it is more aerotolerant than *S. saccharolyticus*. Thus, differentiating *S. saccharolyticus* from other anaerobes is difficult when growth has only been attempted in anaerobic conditions. Like finegoldiae and

Table 1. Differentiation of anaerobic Gram-Positive cocci.

Genus	G + C%	Peptidoglycan (Pos3, bridge)	Butyrate production	Capronate production	Peptone as major energy source	Sugar fermented	Sugar required for growth
Genus Anaerococcus gen. nov.	30–35	Lys, D-Glu	+	D	+	w	−
A. prevotii T		Lys, D-Glu	+	−	+	w	−
Genus Peptoniphilus gen. nov.	30–34	Orn, D-Glu	+	−	+	−	−
P. asaccharolyticus T		Orn, D-Glu	+	−	+	−	−
Genus Gallicola gen. nov.	32–34	Orn, D-Asp	−	−	+	−	−
G. barnesiae T		Orn, D-Asp	−	−	+	−	−
Genus Finegoldia	32–34	Lys, Gly	−	−	+	−	−
F. magna T		Lys, Gly	−	−	+	−	−
Genus Micromonas	28–30	Lys, D-Asp	−	−	+	−	−
C. micros T		Lys, D-Asp	−	−	+	−	−
Genus Peptostreptococcus	34–36	Lys, D-Asp	+	+	+	w	−
P. anaerobius T		Lys, D-Asp	+	+	+	w	−
Genus Peptococcus	50–51	Lys, D-Asp	+	+	+	−	−
P. niger T		Lys, D-Asp	+	+	+	−	−
Genus Ruminococcus	39–48	m-DAP, none	−	−	−	+	+
R. flavefaciens T		m-DAP, none	−	−	−	+	+
Genus Coprococcus	39–42	m-DAP, none	+	−	−	+	+
C. eutactus T		m-DAP, none	+	−	−	+	+
Genus Sarcina	28–31	LL-DAP, Gly	D	−	−	+	+
S. ventriculi T		LL-DAP, Gly	−	−	−	+	+

D, different among species; a, reference; T, type species; w, weak acid produced; m- and LL-DAP, meso- and LL-diastereomers of the dibasic amino acid, diaminopimelic acid.

micromonades, *Staphylococcus saccharolyticus* produces acetic acid as a major metabolic product from PYG medium and thus must be differentiated from them. Simple biochemical tests, such as the nitrate reduction test and the urease test, are useful in this regard.

Differentiation of Anaerobic Cocci

Table 1 shows genus-level features used to differentiate anaerobic Gram-positive cocci. Anaerococci, peptoniphili, gallicolae, finegoldiae, micromonades, peptostreptococci, and peptococci use peptones as their major energy sources, and carbohydrates are not required for their growth. On the other hand, ruminococci, coprococci and sarcinae require carbohydrate for their confluent growth. Species-level differentiation of peptone-digesting organisms that require carbohydrates for their growth are shown in Tables 2 and 3. Species in Table 2 can be differentiated from ruminococci, coprococci and sarcinae because peptones (but in most cases not carbohydrates) are their major energy sources. *Peptoniphilus* spp. can be differentiated from peptostreptococci and peptococci because *Peptoniphilus* produces butyrate but not capronate from PYG medium.

 Peptococcus asaccharolyticus is often isolated from human clinical specimens and are a part of normal flora of the human vagina and gastrointestinal tract (Bartlett, 1990; Hill et al., 1995;

Hillier et al., 1993; Hillier and Moncla, 1991). However, this species contains many different genotypes (Ezaki et al., 1983; Ezaki et al., 1992). This suggests that the biology and pathogenicity of this species is not well defined. The identification of *P. asaccharolyticus* is controversial. *Peptoniphilus asaccharolyticus* should be differentiated from other indole-positive species *P. hydrogenalis*, *A. indolicus*, and *A. harei*. In spite of the phenotypic similarity between *A. indolicus* and *P. asaccharolyticus* (both are strongly saccharolytic and have weak proteolytic enzyme activities), the coagulase and positive nitrate reduction tests often help differentiate the two species. However, some *P. asaccharolyticus* reduce nitrate.

Strains of *P. hydrogenalis* are strongly saccharolytic and produce indole. Their proteolytic enzyme activity is very weak as shown in Table 2. They were isolated from human feces and vaginal discharge (Ezaki et al., 1992).

Peptococcus indolicus is isolated from lesions and abscesses of sheepherders, rarely from other human specimens (Bourgault and Rosenblatt, 1979), and from cows with bovine summer mastitis (Hoi-Sorenson, 1973; Madsen et al., 1992). Although biochemically similar to *P. asaccharolyticus*, *P. indolicus* produces coagulase, reduces nitrate to nitrite, and forms propionate from lactate (Holdeman et al., 1977). Species are proteolytic and do not ferment sugars.

Peptococcus lacrimalis was first isolated from the human lacrimal gland (Li et al., 1992) and

Table 2. Differentiation of newly reclassified anaerobic Gram positive cocci, which use peptones as major energy source.

Organism	Accession No. of 16S rDNA	Mol% G+C	Major Terminal FA	Production of				Sugar fermentation				Production of saccharolytic and proteolytic enzymes									
				Indole	Urease	ALP	Coagulase	Glucose	Lactose	Raffinose	Mannose	αGAL	βGAL	αGLU	βGUR	ArgA	ProA	PheA	LeuA	PyrA	HisA
Genus *Anaerococcus* gen. nov.		26–34																			
A. prevotii T	D14139	29–33	B	–	d	–	–	d	–	+	+	+	–	+	+	+	–	–	–	+	+
A. tetradius	D14142	30–32	B	–	+	–	–	d	–	+	+	–	–	+	+	+	–	w	+	w	w
A. lactolyticus	D14154	30–34	B	–	+	–	–	+	+	–	+	–	+	–	–	+	–	–	–	–	–
A. hydrogenalis	D14140	26–31	B	+	d	d	–	+	+	+	+	–	–	d	–	–	–	–	–	–	–
A. octavius	Y07841	30–34	B, C	–	–	–	–	+	–	+	+	–	–	–	–	+	+	–	–	w	–
A. vaginalis	D14146		B	d	–	d	–	+	–	–	d	–	–	–	–	+	–	–	+	–	+
Genus *Peptoniphilus* gen. nov.		25–34																			
P. asaccharolyticus T	D14138	31–32	B	d	–	–	–	–	–	–	–	–	–	–	–	+	–	–	d	–	w
P. lacrimalis	D14141	30–34	B	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	+
P. harei	Y07839	25	B	d	–	–	–	–	–	–	–	–	–	–	–	+	–	–	d	–	+
P. ivorii	Y07840	29	B	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
P. indolicus	D14147	32–34	B	+	–	+	+	–	–	–	–	–	–	–	–	+	–	+	+	–	+
Genus *Gallicola* gen. nov.																					
G. burnesiae T	AB038361	32–34	A, B	w	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Genus *Finegoldia*																					
F. magna T	D14149	32–34	A	–	–	d	–	–	–	–	–	–	–	–	–	+	–	–	+	+	d
Genus *Micromonas*																					
M. micros T	D14143	27–28	A	–	–	+	–	–	–	–	–	–	–	–	–	+	+	–	+	+	+
Genus *Peptostreptococcus*																					
P. anaerobius T	L04168	33–34	iC, iV	–	–	–	–	+	–	–	w	–	–	+	–	–	+	–	–	–	–
Genus *Peptococcus*																					
P. niger T	X55797	50–51	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Symbols: +, present; –, absent.

Abbreviations: E33FA, fatty acid; B, butyrate; C, capronate; iC, iso-capronate; iV, iso-valerate; A, acetate; ALP, alkaline phosphatase; αGAL, α-galactosidase; βGAL, β-galactosidase; αGLU, α-glucosidase; βGUR, β-glucuronidase; ArgA, arginine arylamidase (AMD); ProA, proline AMD; PheA, phenylalanine AMD; LeuA, leucine AMD; PyrA, pyroglutamyl AMD; HisA, histidine AMD; d, different among strains; T, type species; w, weak.

Table 3. Biochemical characteristics of carbohydrate requiring Gram positive anaerobic cocci.

Organism	Accession No. of 16S rDNA	Mol% G+C	Major terminal FA	Fermentation of										Esculin hydrolysis
				Arabinose	Cellobiose	Glucose	Lactose	Mannose	Maltose	Mannitol	Raffinose	Sucrose	Xylose	
Genus Ruminococcus		37–48												
R. flavefaciens T	X83430	39–44	A, F, S	–	+	–	+	–	–	–	–	–	–	+
R. albus	X85098	43–46	A, F	–	+	+	+	+	–	–	–	+	–	+
R. bromii	X85099	39–40	A	–	–	+	–	–	+	–	–	–	–	–
R. callidus	X85100	42	S, A	–	+	+	+	w/–	+	–	+	+	w/–	+
R. gnavus	D14136	41	A, F	+	–	+	–	–	+	–	+	+	+	+
R. hansenii	D14155	37–38	L, A	–	+	+	+	–	+	–	+	–	+	d
R. hydrogenotrophicus	X95624	45	A,	–	–	+	ND	d	ND	–	ND	–	ND	ND
R. lactaris	L76602	43	A, F	–	+	+	+	w/–	d	+	+	+	–	d
R. obeum	X85101	45	A	+	–	+	+	+	+	–	+	–	+	ND
R. palustris	ND	47–48	ND	–	+	+	+	+	+	+	+	+	–	+
R. productus	D14144	44–45	L, A	+	+	+	+	+	+	+	+	+	+	ND
R. schinkii	X94965	46–47	A	+	+	+	ND	+	+	ND	+	–	+	ND
R. torques	L76604	43	L, A	–	–	+	+	w/–	w	–	–	–	–	d
Genus Coprococcus		39–42												
C. eutactus T	D14148	41	F, B, L, A	–	+	+	+	+	+	–	+	+	–	+
C. catus	AB038359	39–41	B, P, A	–	–	w/–	–	–	–	+	–	–	–	–
C. comes	ND	40–42	L, B, A	+	–	+	+	w/–	+	d	+	+	+	d
Genus Sarcina		29–31												
S. ventriculi T	X76649	31–31	A, F	–	–	–	+	+	+	–	+	+	–	+
S. maxima	X76650	29–24	B, A	d	–	+	–	+	+	ND	ND	+	+	ND

Symbols: +, present; –, absent.
Abbreviations: d, different among strains; ND, no data; FA, fatty acids; A, acetate; F, formate; S, succinate; L, lactate; P, propionate; T, type species.

identified as "*P. prevotii*" (now called *A. prevotii*) because it did not produce indole. However, 16S rRNA and DNA/DNA hybridization analyses revealed a new species. Its biochemical characteristics are similar to anaerococci, except that it does not ferment carbohydrate.

Peptococcus harei was first reported as "*Peptostreptococcus harei*" (Murdoch and Mitchelmore, 1991) and isolated from various skin abscesses (Murdoch et al., 1997). Some strains produce indole and catalase. All strains are asaccharolytic and moderately proteolytic.

Peptococcus ivorii, first isolated from human leg ulcers and clinical specimens (Murdoch et al., 1997), is asaccharolytic and weakly proteolytic. Because it produces minor amounts of isovaleric acid, isolates can be misidentified as *Peptostreptococcus anaerobius*.

Anaerococcus spp. produce weak acids from several carbohydrates but they also use peptones as their energy source. As a result, they produce butyrate from PYG medium.

Anaerococcus prevotii were recognized as the only indole-negative and butyrate-positive anaerobic Gram-positive cocci until the early 1990s (Holdeman et al., 1986) when six other species (isolated from vaginal discharges, and ovary and skin abscesses) were described (Ezaki et al., 1983; Murdoch et al., 1997; Ezaki et al., 2000) as saccharolytic and proteolytic. *Anaerococcus tetradius*, previously classified as "*Gaffkya anaerobica*," was transferred to the genus *Peptostreptococcus* (Ezaki et al., 1983). However, after 16S rRNA analysis, its reclassification as a member of the genus *Anaerococcus* (Ezaki et al., 2000) was proposed. Like *A. prevotii*, *A. tetradius* has saccharolytic and proteolytic activity. Only a few biochemical tests are able to differentiate the two species. *Anaerococcus tetradius* is a vaginal microorganism, also isolated from ovarian and other abscesses.

Anaerococcus vaginalis was isolated from vaginal discharges (Li et al., 1992). A weakly saccharolytic species, its proteolytic enzymes and sugar fermentation patterns are generally useful for differentiating these organisms from other anaerococci.

Anaerococcus hydrogenalis is a strongly saccharolytic species. It is also isolated from vaginal discharge and stool from humans (Ezaki et al., 1990). Gas production from PYG is very strong and often a test tube with rubber stopper will explode. Indole production is positive.

Anaerococcus lactolyticus, isolated from the human vagina and the gastrointestinal tract (Li et al., 1992), is weakly saccharolytic, weakly proteolytic and urease positive. *Anaerococcus octavius*, isolated from the nasal cavity (Murdoch et al., 1997), is weakly saccharolytic and produces a small amount of capronate from PYG.

Finegoldia magna is an anaerobic cocci frequently isolated from human clinical specimens (Bourgault et al., 1980; Brook, 1981; Brook, 1988; Brook, 1989; Brook, 1995; Brook, 1996), including ovarian, peritoneal and skin abscesses (Brook, 1988; Hunter and Chow, 1988; Finegold, 1995). The organism was recently transferred from the genus *Peptostreptococcus* (Murdoch and Shah, 1999). *Finegoldia magna* does not produce butyrate but rather acetate as a major metabolic end product from PYG. No carbohydrates are fermented. Although phenotypically related to *Micromonas micros*, *F. magna* cells are 0.8 to 1.9 µm and *M. micros* cells are 0.3 to 0.7 µm.

Micromonas micros was once a member of the genus *Peptostreptococcus* (Murdoch and Shah, 1999). It is among the normal flora of the oral cavity (Murdoch et al., 1988). All strains have a strong alkaline phosphatase activity (Ezaki and Yabuuchi, 1985), whereas only some *F. magna* strains have alkaline phosphatase activity, which is usually very weak. Strains of *F. magna* from various sources were genetically identical (T. Ezaki, unpublished observation), but *M. micros* reportedly has two morphotypes (Van Dalen, 1993).

Peptostreptococcus anaerobius now is the only species in the genus. It is isolated from stool samples and the vagina. The cells are usually coccobacilli and highly pleomorphic. Isocapronate and isovalerate are major metabolic end products. On the basis of the coccobacillus morphology and pattern of metabolic products, it is often misidentified as clostridium. The species is also clustered in the clostridium XI group (Collins et al., 1994).

Peptococcus niger has been isolated from human urine (Hall, 1930), the umbilicus (Wilkins et al., 1975) and the vagina (Marui, 1981). Initially black colonies form on blood agar plates, but after successive culture on a blood agar, gray colonies form, that is to say if they form at all. Capronate production from PYG (Holdeman et al., 1986) is a characteristic. Sugars are not fermented and proteolytic enzyme activity is not detected.

Gallicola barnesiae originally was isolated from chicken feces (Schiefer-Ullrich and Andreesen, 1985). The other sources are not clearly identified. It is asaccharolytic and feebly indole positive.

Differentiation of ruminococci, coprococci and sarcinae

Differential characteristics of ruminococci, coprococci and sarcinae are shown in Table 3.

Growth of ruminococci and coprococci is remarkably enhanced by rumen fluid (Holdeman and Moore, 1974). The two genera can be

differentiated on the basis of formate, acetate and lactate, which are produced from PYG by ruminococci and butyric acid, which is produced from PYG by coprococci. Ruminococci are divided into two phylogenetic groups (Fig 2). The second group of ruminococci and the coprococci are in the same phylogenetic group. Species in the genus *Ruminococcus* and *Coprococcus* are normal flora of the human and animal gastrointestinal tract (Bryant, 1986).

Ruminococus productus (formerly *Peptostreptococcus productus*; Ezaki et al., 1994) ferments various carbohydrates and produces lactate and acetate from PYG.

Ruminococcus hansenii was once classified as *Streptococcus hanseni* (Holdeman and Moore, 1974) because the major metabolic end product was lactate. However, the species (an obligate anaerobe) was phylogenetically a member of the genus *Ruminococcus* (Ezaki et al., 1994). It has strong saccharolytic activities and occasionally is isolated from human clinical specimens (Marui, 1981).

Sarcinae have characteristic packet morphology and can form spores. Organisms have been found in the gastric contents and feces of patients with gastric disorders (Crowther, 1971), and they are common in the soil and the stool of vegetarians (Ezaki et al., 1994; Crowther, 1971).

Sarcina maxima and *S. ventriculi* can be differentiated by their metabolic end products, the former producing butyric acid and the latter formate and acetate.

Molecular Identification of Anaerobic Cocci

Biochemical differentiation of anaerobic cocci is technically demanding and often unsuccessful because of their fastidious growth in chemically defined media. On the other hand, almost all 16S rDNA sequences of anaerobic cocci have been determined. Determining the phylogenetic position of anaerobic cocci (Fig. 3) is much easier than biochemical characterization. The following scheme is also applicable for molecular identification of all bacteria. First, determine the partial 16S rDNA sequence of unidentified anaerobic cocci. Amplify the 16S rDNA with two universal primers (16S-up: 5′-AGA-GTT-TGA-TC(A or C)-TGG-CTC-AG-3′ and 16S-down: 5′-CAG-C (A or C)G-CCG-CGG-TAA-T-3′). A PCR product of approximately 500 bp will be amplified. For the phylogenetic analysis, a full-range sequence is essential but a partial sequence is usually enough for identification. The next step is FAST or Basic Local Alignment Search Tool (BLAST) analysis through the DNA Databank of Japan (DDBJ), Gene Bank or European Molecular Biology Laboratory (EMBL) to find related sequences. If the organism is truly an
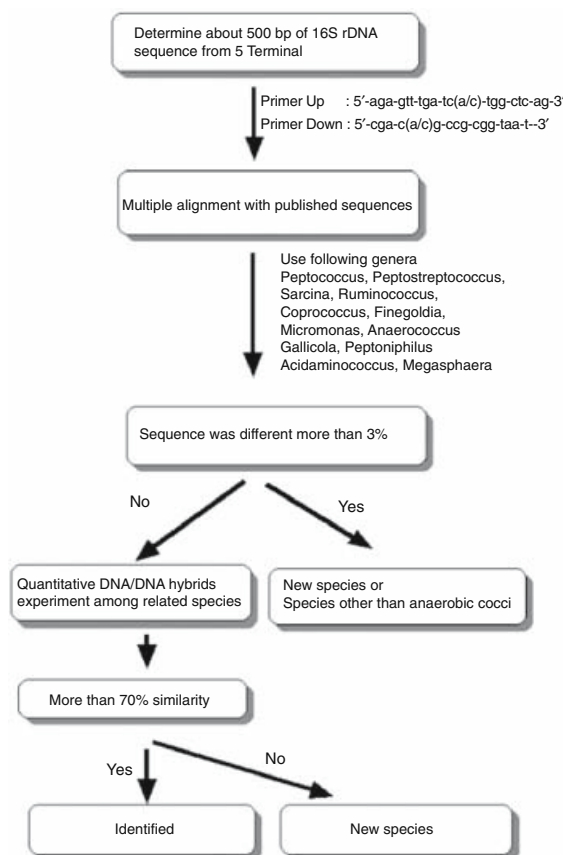
Fig. 3. Identification scheme for anaerobic cocci.

anaerobic cocci, sequences of the species listed in Table 1 would be picked up from the above databases.

After preparing a data set of the related species, run multiple alignments with software Clustal W (Thompson, 1994). If the similarity values between the examined strain and the closest species are smaller than 97%, the examined strain might be a new species. If the similarity value is higher than 97%, then perform quantitative DNA/DNA hybridization among related species (Stackebrandt and Goebel, 1994a). Quantitative DNA/DNA hybridization values higher than 70% among the closest strains means that the two species are taxonomically identical (Johnson, 1984; Wayne et al., 1987). Simple quantitative DNA/DNA hybridizations can be done in 96-well microtiter plates (Ezaki et al., 1989; Shatha et al., 1993; Cristensen et al., 2000).

Antibiotic Susceptibility

Anaerobic cocci usually are sensitive to all the antibiotics commonly used to treat anaerobic infections (Finegold and George, 1989; Garcia-Rodriguez et al., 1995; Murdoch, 1998). Rou-

tine susceptibility testing for anaerobes is not required. Most evidence suggests that *P. asaccharolyticus*, *F. magna*, *M. micros* and *A. prevotii* are almost always susceptible to the penicillins (Bowker et al., 1996; Johnson, 1993). Of the cephalosporins, cefoxitin is probably the most effective (Murdoch, 1998). Cefotaxime often appears to have borderline activity (Murdoch, 1998; Citron et al., 1995; Panichi et al., 1990).

Metronidazole usually is effective (Watt and Smith, 1990). Microaerophilic streptococci, often misidentified as true anaerobic cocci, are resistant to metronidazole. For this reason, true anaerobic cocci are often misunderstood to be resistant to metronidazole. Nevertheless, some isolates of *Ps. anaerobius* are moderately resistant to metronidazole (Bowker et al., 1996).

Erythromycin, clarithromycin and azithromycin are probably not effective enough to be recommended for treatment (Brudoch, 1998; Sanchez et al., 1992). Teicoplanin and vancomycin are effective (Greenwood and Palfreyman, 1987). Several studies indicate that first generation quinolones have only moderate antibacterial activity against the anaerobic cocci; however, a recent study indicates that anaerococci, peptoniphili, finegoldia, and micromonades are consistently highly susceptible to trovafloxacin and clindafloxacin (Murdoch, 1998). Only isolates of *Ps. anaerobius* are often borderline and resistant (Murdoch, 1998). Carbapenems are extremely active, and new quinolones are also very effective.

Wilkins and Chalgren (1976) worked out techniques for the determination of minimum inhibitory concentration (MIC). The medium developed supports the growth of anaerococci, peptoniphili, finegoldiae, and micromonades, but most isolates of ruminococci, coprococci and sarcinae do not grow on the medium. Standard procedures to determine antibiotic susceptibility of anaerobes were published (Finegold and NCCLS, 1988; US standard, National Committee for Clinical Laboratory Standards, 1990; UK standard, British Society for Antimicrobial Chemotherapy, 1991).

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CHAPTER 1.2.27

The Order Haloanaerobiales

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Introduction

Bottom sediments of hypersaline lakes and lagoons may be expected to support a rich community of anaerobic halophilic bacteria, as the solubility of oxygen in hypersaline brines is low and the amounts of organic matter available are often high (Oren, 1988). Therefore, it is surprising that the first records of obligatory anaerobic fermentative bacteria growing at salt concentrations of 10–20% and higher appeared only in the early 1980s, when *Haloanaerobium praevalens* was isolated from the bottom sediments of the Great Salt Lake in Utah (Zeikus, 1983; Zeikus et al., 1983) and *Sporohalobacter lortetii* and *Halobacteroides halobius* were discovered in Dead Sea sediments (Oren, 1983; Oren et al., 1984b). *Haloanaerobium praevalens* may be similar to "*Bacteroides halosmophilus*," isolated by Baumgartner, 1937 from solar salt and from salted anchovies, but unfortunately no cultures of that isolate have been preserved.

The fermentative obligatory anaerobic halophilic Bacteria appear to be a phylogenetically coherent group (Rainey et al., 1995). The order Haloanaerobiales was created to accommodate these halophilic anaerobes. At the time of writing (January 1999), 19 species had been described and classified in two families, the Haloanaerobiaceae (Oren et al., 1984a; Rainey et al., 1995) and the Halobacteroidaceae (Rainey et al., 1995). This chapter presents these species and discusses their properties. The group was earlier reviewed by Lowe et al., 1993, Ollivier et al., 1994, and Oren, 1986a, Oren, 1990, Oren, 1993a, Oren, 1993b.

Phylogeny

Phylogenetic analysis based on 16S rRNA gene sequences has shown that the 19 obligatory anaerobic halophilic bacteria validly described thus far, may be classified in a single order, the Haloanaerobiales (Rainey et al., 1995), with two families, the Haloanaerobiaceae (Oren et al., 1984a) and the Halobacteroidaceae (Rainey et al., 1995) (Table 1, Fig. 1). Figures 2–3 present

micrographs and electron micrographs of selected species.

All isolates share a low content of G+C in their DNA. With the exception of the thermophilic *Halothermothrix orenii*, which has a G+C content of 39.6 mol%, all species have G+C contents between 27 and 36.9 mol%.

Based on 16S rRNA gene sequences, the halophilic anaerobic bacteria should be classified in the domain Bacteria within the phylum of the Gram-positive bacteria. They form a coherent cluster close to the bifurcation point that separates the Actinomycetes subphylum and the *Bacillus/Clostridium subphylum*. Sequences of the halophilic anaerobes contain all of the few signature nucleotides that have been defined as characteristic of members of the *Bacillus/Clostridium subphylum*, while they lack any of the Actinomycetes-specific nucleotides. Therefore, the halophilic anaerobes were suggested to be in the *Bacillus/Clostridium subphylum* (Patel et al., 1995; Rainey et al., 1995; Tourova et al., 1995). The phylogenetic affiliation of *Haloanaerobium praevalens* with the *Bacillus/Clostridium* group was confirmed by the amino acid sequence of its ribosomal A-protein (Matheson et al., 1987). The location of the branching point of the halophilic anaerobes close to the root of this subphylum is further evidence that certain descendants of the ancestors of the Gram-positive bacteria still maintained their Gram-negative wall type, as is the case with *Sporomusa* and relatives. The deep branching justifies classification in a separate order, and thus the order Haloanaerobiales was created (Rainey et al., 1995). Table 2 presents the signature nucleotides defining the Haloanaerobiales within the *Bacillus/Clostridium subphylum* of the Gram-positive Bacteria. The 16S rRNA signature nucleotides defining the two families are shown in Table 3.

Taxonomy

The properties of the species assigned to the families Haloanaerobiaceae and Halobacteroidaceae are summarized in Tables 4–5, respectively.

All species show a negative Gram stain reaction. Electron micrographs of thin sections generally show a typical Gram-negative type of cell wall with a prominent outer membrane and periplasmic space (see e.g., Figs. 3B, D, E, and G). The presence of meso-diaminopimelic acid in the peptidoglycan has been documented in *Haloanaerobium saccharolyticum* (Zhilina et al., 1992b).

A number of species of the Haloanaerobiales have been shown to produce heat-resistant endospores. These include *Sporohalobacter lortetii* (Oren, 1983), *Orenia marismortui* (Oren et al., 1987), and *Natroniella acetigena* (Zhilina et al., 1996). When initially isolated, *Acetohalobium arabaticum* produced sometimes spores, but with continued cultivation, sporulation was no longer observed (Zavarzin et al., 1994).

Habitat

Members of the Haloanaerobiales have been isolated from a wide variety of anaerobic hyper-

Table 1. Taxonomic structure of the order Haloanaerobiales.

Order Haloanaerobiales	
Family Haloanaerobiaceae	Family Halobacteroidaceae
Genera and species	Genera and species
Haloanaerobium	*Halobacteroides*
praevalens[T]	*halobius*[T]
alcaliphilum	*elegans*
acetoethylicum	*Acetohalobium*
salsuginis	*arabaticum*[T]
saccharolyticum	*Haloanaerobacter*
subsp. *saccharolyticum*	*chitinovorans*[T]
subsp. *senegalensis*	*lacunaris*
congolense	*salinarius*
lacusrosei	*Orenia*
kushneri	*marismortui*[T]
Halothermothrix	*Sporohalobacter*
orenii[T]	*lortetii*[T]
Halocella	*Natroniella*
cellulolytica[T]	*acetigena*[T]

[T] = type species of the genus.
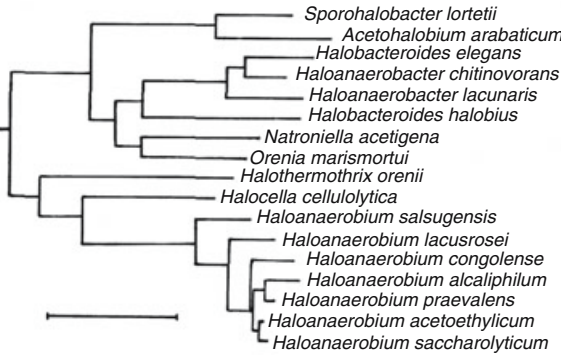
Fig. 1. Phylogenetic tree, showing the phylogenetic position of selected members of the *Haloanaerobiales*. Scale bar represents 5 nucleotide exchanges per 100 nucleotides. Modified from Zhilina et al., 1997.
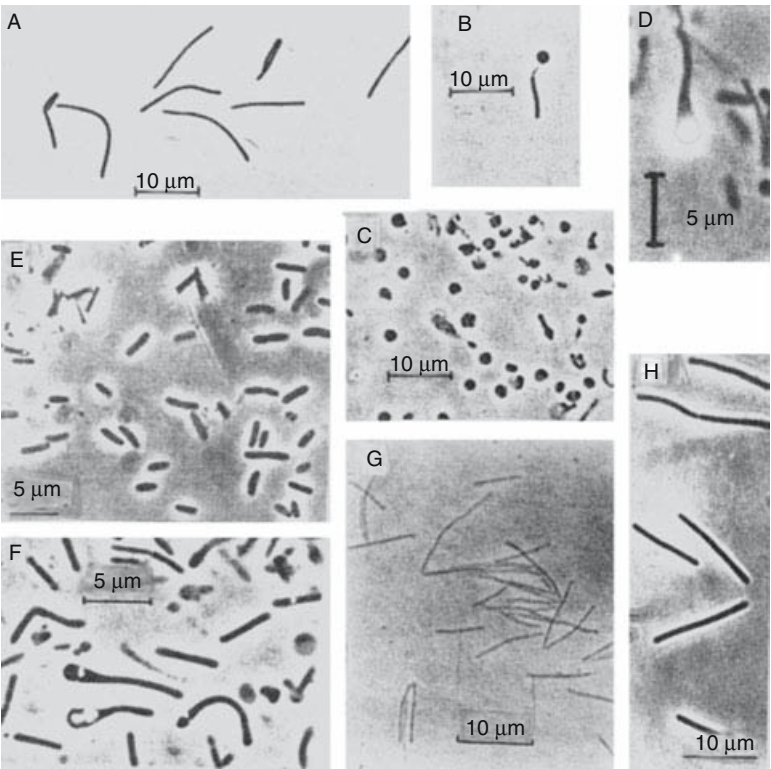
Fig. 2. Phase-contrast micrographs of members of the *Haloanaerobiales*. Scale bar represents 5 nucleotide exchanges per 100 nucleotides. Modified from Zhilina et al., 1997. (A,B,C) young, senescent, and old cells of *Halobacteroides halobius*; (D) *Orenia marismortui*; (E) *Haloanaerobium alcaliphilum*; (F) *Sporohalobacter lortetii*; (G) *Halothermothrix orenii*; (H) *Natroniella acetigena*. Figures were derived from Oren et al., 1984b, Oren et al., 1987, Tsai et al. (1995), Oren, 1983, Cayol et al., 1994b and Zhilina et al., 1996 respectively; reproduced with permission.
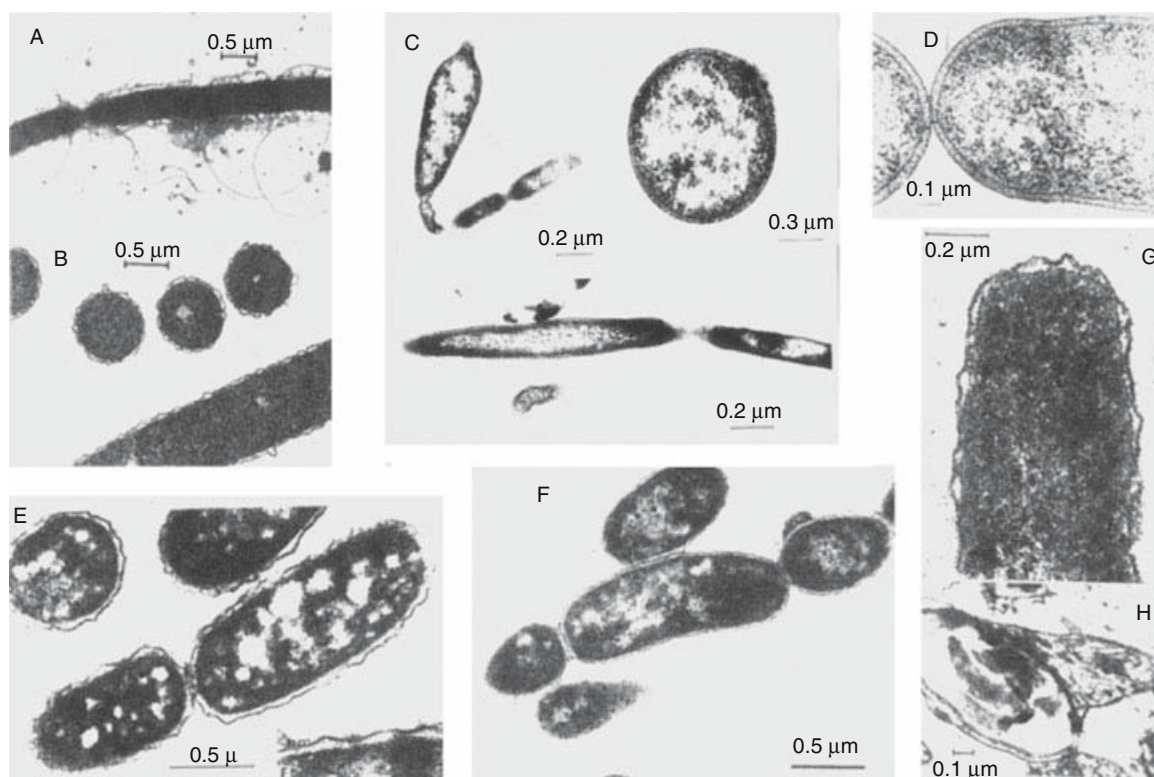
Fig. 3. Electron micrographs of members of the Haloanaerobiales: (A,B) *Halobacteroides halobius*; (C) *Halothermothrix orenii*; (D) *Haloanaerobium saccharolyticum* subsp. *senegalensis*; (E) *Haloanaerobium saccharolyticum* subsp. *saccharolyticum*; (F) *Haloanaerobium lacusrosei*; (G,H) *Sporohalobacter lortetii*. Figures were derived from Oren et al., 1984b, Cayol et al., 1994b, Cayol et al., 1994a, Cayol et al., 1995, and Oren, 1983 respectively; reproduced with permission.

Table 2. 16S rRNA signature nucleotides defining the Haloanaerobiales within the *Bacillus/Clostridium subphylum* of Gram-positive bacteria (from Rainey et al., 1995; reproduced with permission).

Position E. coli nomenclature	Haloanaerobiales (>90% of species)	Majority of the members of the *Clostridium/Bacillus* subphylum (>90%)
94	one-base insertion	no insertion
771–808	U-A	G-C
772–807	R-Y	U-A
784–798	G-C	A-U
890	mainly U	G
1059–1198	mainly C-G	U-A
1115	mainly C	U
1415–1485	Y-R	G-U

Y = pyrimidine; R = purine.

saline environments. Fermentative halophilic bacteria have been recovered from: the Great Salt Lake, in Utah (Tsai et al., 1995; Zeikus et al., 1983); Salton Sea in California (Shiba, 1991; Shiba and Horikoshi, 1988; Shiba et al., 1989); the Dead Sea (Oren, 1983; Oren et al., 1984b; 1987); a hypersaline sulfur spring on the shore of the Dead Sea (Oren, 1989); oil wells and petro-leum reservoir fluids (Bhupathiraju et al., 1991, 1993, 1994, 1999; Ravot et al., 1997; Rengpipat et al., 1988a); hypersaline lakes and lagoons in the Crimea (Simankova et al., 1993; Zhilina and Zavarzin, 1990a; Zhilina et al., 1991, 1997) and in Senegal (Cayol et al., 1994a, 1995); saltern ponds in California (Liaw and Mah, 1992) and France (Mouné et al., 1999); a hot hypersaline lake in Tunisia (Cayol et al., 1994b); and the alkaline hypersaline lakes, Magadi, in Kenya (Shiba and Horikoshi, 1988; Zhilina et al., 1996) and Big Soda Lake in Nevada (Shiba and Horikoshi, 1988; Shiba et al., 1989). Thus, the range of habitats includes both thalassohaline and athalassohaline environments.

Isolation

Any anoxic reducing medium containing high salt concentrations (5–25%) and containing a suitable carbon source is a potential enrichment and growth medium for anaerobic halophilic bacteria. A variety of such media have been used for the growth and isolation of different members of the *Haloanaerobiales*. Table 6 presents a selection.

Table 3. 16S rDNA signature nucleotides defining the two families within the order Haloanaerobiales (from Rainey et al., 1995; reproduced with permission).

Position (*E. coli* nomenclature)	Haloanaerobiaceae	Halobacteroidaceae
70	C	A
73	Y	A
75	C	G
Variable region I (73–97)	Long stem	Short stem
90	U	A
98	G	U
100	Y	R
135	U	C
Variable region II (184–193)	Long stem	Short stem
233	U	C
241–285	A-U	C-G
242–284	G-C	C-G
274	G	A
284	C	G
291–309	U-A	C-G
294–303	U-A	C-G
293–304	G-C	A-U
353	A	U
453	C	A
459–473	U-A/A-U	G-C
467	A	U
457–475	G-Y	Y-R
479	C	U
589–650	R-U	U-A
590–649	U-A	C-G
591–648	A-U	U-A
657–749	U-A	R-Y
896–903	U-A	C-G
943–1340	U-A	C-G
986–1219	G-C	A-U
987–1218	A-U	G-C
1168	ND	A/C
1210	U	C
1245–1292	R-Y	U-A

Y = pyrimidine; R = purine; ND = no data available.

The Haloanaerobiales are obligate anaerobes. Though they are not extremely sensitive to molecular oxygen, the use of strict anaerobic techniques is recommended, including boiling the media under nitrogen or nitrogen-CO_2 (80 : 20), and adding reducing agents such as cysteine, dithionite, or ascorbate to the boiled media.

The existence of heat-resistant endospores has been exploited in a selective enrichment procedure for *Halobacteroides halobius*-like bacteria, based on negative selection by pasteurization of the inoculum for 10–20 min at 80–100°C (Oren, 1987).

Identification

Identification of isolates and their assignment to one of the recognized genera and species of the Haloanaerobiales should be based preferentially on the determination of their 16S rRNA gene sequences. In addition, phenotypic characterization should be performed and the properties of the strains should be compared with those of the described species (Tables 4–5). Their ability to grow on different carbohydrates and other simple compounds can be used often to differentiate between the species. Identification should be based on a full phenotypic characterization and not on a few selected properties only. For example, chitinolytic activity is a poor characteristic for differentiating *Haloanaerobacter chitinovorans* from other anaerobic halophiles, as only one of the two strains described, which share 92.3% DNA-DNA homology, degrades chitin (Liaw and Mah, 1992; Rainey et al., 1995).

The members of the Haloanaerobiales can be divided into three groups on the basis of the substrates fermented. Most species are able to ferment a variety of carbohydrates. One species, *Sporohalobacter lortetii*, seems to be primarily an amino acid fermenter, and sugars are used only poorly (Oren, 1983). Serine can be used as electron donor in the Stickland reaction by *Haloanaerobacter salinarius* with glycine betaine as electron acceptor, with formation of acetate, trimethylamine, CO_2, and NH_3 (Mouné et al., 1999). Finally, the neutrophilic *Acetohalobium arabaticum* and the alkaliphilic *Natroniella acetigena* are homoacetogens, which do not use carbohydrates but grow chemoheterotrophically on substrates such as lactate, ethanol, pyruvate, glutamate, propionate, and glycine betaine, or chemoautotrophically on hydrogen + carbon dioxide (*Acetohalobium* only), producing acetate (Zavarzin et al., 1994; Zhilina and Zavarzin, 1990a, 1990b; Zhilina et al., 1996).

The property of endospore formation may be expressed only under special conditions, especially on solid growth media or in nutrient-poor liquid media (Oren, 1983; Oren et al., 1987). Certain representatives make spores only rarely, e.g., *Natroniella acetigena* (Zhilina et al., 1996) and *Acetohalobium arabaticum*. The latter is reported to produce spores when originally isolated but loses this property upon continued cultivation (Zavarzin et al., 1994). A phenotypic test, which may be correlated with the phylogenetic position of the Haloanaerobiaceae within the phylum of the Gram-positive Bacteria and with the ability to form endospores, is the hydrolysis of the D-isomer of N′-benzoyl-arginine-p-nitroanilide (BAPA). Four representatives of the Haloanaerobiales were tested for D-BAPA and

Table 4. The validly described species belonging to the family Haloanaerobiaceae and their properties.

	Haloanaerobium praevalens	Haloanaerobium alcaliphilum	Haloanaerobium acetobutylicum	Haloanaerobium salsugo	Haloanaerobium saccharolyticum subsp. saccharolyticum	Haloanaerobium saccharolyticum subsp. senegalensis	Haloanaerobium congolense	Haloanaerobium lacusroseus	Haloanaerobium kushneri
Former designation			Halobacteroides acetoethylicus	Haloanaerobium salsugo	Haloincola saccharolyticus (saccharolytica)				
Type strain	DSM 2228	DSM 8275	DSM 3532	ATCC 51327	DSM 6643	DSM 7379	DSM 11287	DSM 10165	ATCC 700103
Cell size	0.9–1.1×2.0–$2.6\mu m$	0.8×3.3–$5\mu m$	0.4–0.7×1–$1.6\mu m$	0.3–0.4×2.6–$4\mu m$	0.5–0.7×1–$1.5\mu m$	0.4–0.6×2–$5\mu m$	0.5–1×2–$4\mu m$	0.4–0.6×2–$3\mu m$	0.5–0.8×0.7–$3.3\mu m$
Morphology	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Motility	–	+	+	–	+	+	–	+	+
Flagella	–	Peritrichous	Peritrichous	–	Peritrichous	Peritrichous	–	Peritrichous	Peritrichous
Spores	–	–	–	–	–	–	–	–	–
Spheroplasts	–	NR	–	NR	–	NR	NR	NR	NR
Gas vesicles	NR	NR	NR	NR	NR	NR	NR	NR	NR
NaCl range	2–30%	2.5–25%	5–22%	6–24%	3–30%	5–25%	4–24%	7.5–34%	9–18%
NaCl optimum	13%	10%	10%	9%	10%	7.5–12.5%	10%	18–20%	12%
pH range	6.0–9.0	5.8–10.0	5.4–8.0	5.6–8.0	6.0–8.0	6.3–8.7	6.3–8.5	NR	6.0–8.0
pH optimum	7.0–7.4	6.7–7.0	6.3–7.4	6.1	7.5	7.0	7.0	7.0	6.5–7.5
Temperature range	5–50°C	25–50°C	15–45°C	22–51°C	15–47°C	20–47°C	20–45°C	20–50°C	20–45°C
Temperature optimum	37°C	37–40°C	34°C	40°C	37–40°C	40°C	42°C	40°C	35–40°C
Doubling time	4h	3.3h	7.5h	9h	3.9h	4.2h	2.5h	2.4h	7.3h
Carbohydrates utilized	+	+	+	+	+	+	+	+	+
End products of fermentation	Acetate, Butyrate, Propionate, H_2, CO_2	Acetate, Butyrate, Lactate, H_2, CO_2	Acetate, H_2, CO_2	Acetate, Ethanol, H_2, CO_2	Acetate, H_2, CO_2	Acetate, H_2, CO_2	Acetate, H_2, CO_2	Acetate, Ethanol, H_2, CO_2	Acetate, Ethanol, H_2, CO_2
Major fatty acids[1]	14:0 16:0 16:1	NR	14:0 16:0 16:1	14:0 16:0 16:1 17:0 $_{GxG}$	15:1 16:0 16:1	14:0 16:0 15:1 16:1	NR	NR	14:0 16:0 16:1
DNA mol% G+C	27–28	31.0	32.0	34.0	31.3	31.7	34	32	32.4–36.9
16S rRNA/rDNA accession number	M59123	X81850	X89071	L22890	X89069	X89070	U76632	L39767	U86446
Sample source	Sediment	Sediment	Filter material Offshore oil rig	Petroleum reservoir fluid	Sediment	Sediment	Offshore oil well	Sediment	Petroleum reservoir fluid
Site	Great Salt Lake, Utah, USA	Great Salt Lake, Utah, USA	Gulf of Mexico	Oklahoma, USA	Lake Sivash, Crimea	Lake Retba, Senegal	Congo	Lake Retba, Senegal	Lake Sivash, Crimea
Reference	Zeikus et al., 1983	Tsai et al., 1995	Rengpipat et al., 1988a; Patel et al., 1995; Rainey et al., 1995	Bhupathiraju et al., 1994; Trüper and de'Clari, 1998	Zhilina et al., 1992b; Rainey et al., 1995; Euzéby, 1998	Cayol et al., 1994a; Rainey et al., 1995	Ravot et al., 1997	Cayol et al., 1995	Bhupathiraju et al., 1999

Table 4. *Continued*

	Haloanaerobium species	*Haloanaerobium salsugensis*	*Haloanaerobium acetoethylicum*	*Halobacteroides* species, *Haloanaerobium saccharolyticum*	*Halothermothrix orenii*	*Halocella cellulolytica*
NaCl	130	120	100	100–150	100	150
$MgSO_4 \cdot 7H_2O$	8.8	0.2				
$MgCl_2 \cdot 6H_2O$			0.4	0.33	2.0	3.3
KCl	1.0	0.1		0.33	4.0	0.33
NH_4Cl		1.0	0.9	0.33	1.0	0.33
$CaCl_2 \cdot 2H_2O$		0.2		0.33	0.2	0.33
KH_2PO_4		0.1	0.75	0.33	0.3	0.33
K_2HPO_4			1.5			
$FeSO_4 \cdot 7H_2O$			3mg			
$NaHCO_3$				1.5[a]	5.0[a]	2.5
Na_2CO_3						
$Na_2S \cdot 9H_2O$	0.5[a]	2.5[a]	1.0[a]	0.5[a]	0.2[a]	0.5[a]
Glucose	5.0[a]		5.0[a]	5.0[a]	10[a]	
Chitin						
Microcrystalline cellulose or cellobiose						5.0 or 5.0[a]
Trimethylamine HCl or glycine betaine						
Na-acetate					1.0	
Ethanol						
Yeast extract	10	1.0	3.0[a]			2.0
Trypticase	10		10		0.5	
Peptone			1.0	5.0		
Casamino acids	1.0					
Nutrient broth						
L-glutamic acid						
Vitamin solution[b]	10ml[a]	10ml	5ml	10ml		10ml
Trace element solution	5ml[c]	10ml[c]	9ml[d]	1ml[e]	1ml[c]	1ml[e]
Thioglycolate-ascorbate solution[g]		25ml[a]				
Cysteine HCl	0.5					
Na-dithionite					10mg[a]	
Resazurin	0.5mg	10ml[a]	1mg	2mg	1mg[a]	2mg
NaOH 2N						
PIPES-di-K[h]	1.5					
Final pH[i]	7.1–7.3	9.0	7.2–7.4	7.5	7.0	7.0

Table 5. The validly described species, belonging to the family Halobacteroidaceae, and their properties.

	Halobacteroides halobius	Halobacteroides elegans	Acetohalobium arabaticum	Haloanaerobacter chitinovorans	Haloanaerobacter lacunaris	Haloanaerobacter salinarius	Orenia marismortui	Sporohalobacter lortetii	Natroniella acetigena
Former designation		Halobacteroides halobius			Halobacteroides lacunaris		Sporohalobacter marismortui	Clostridium lortetii	
Type strain	ATCC 35273	DSM 6639	DSM 5501	OCG 229	DSM 6640	DSM 12146, ATCC 700559	ATCC 35420	ATCC 35059	DSM 9952
Cell size	0.5–0.6 × 10–20μm	0.3–0.5 × 2–10μm	0.7–1 × 2–5μm	0.5 × 1.4–8μm	0.5–0.6 × 0.7–1μm	0.3–0.4 × 5–8μm	0.6 × 3–13μm	0.5–0.6 × 2.5–10μm	1–1.2 × 6–15μm
Morphology	Flexible rods	Curved rods	Curved rods	Flexible rods	Slightly curved rods	Flexible rods	Rods	Rods	Rods
Motility	+	+	+	+	+	+	+	+	+
Flagella	Peritrichous	Peritrichous	1–2 Subterminal	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Peritrichous
Spores	$-^2$	+	Rare	–	–	–	+	+	+
Spheroplasts	+	+	NR	+	+	+	–	–	+
Gas vesicles	NR	NR	NR	NR	NR	NR	NR	+	NR
NaCl range	7–19%	10–30%	10–25%	3–30%	10–30%	5–30%	3–18%	4–15%	10–26%
NaCl optimum	9–15%	10–15%	15–18%	12–18%	15–18%	14–15%	3–12%	8–9%	12–15%
pH range	ND	6.5–8.0	5.8–8.4	NR	6.0–8.0	5.5–8.5	NR	NR	8.1–10.7
pH optimum	ND	7.0	7.4–8.0	7.0	6.5–7.0	7.4–7.8	NR	NR	9.7–10.0
Temperature range	30–47°C	28–47°C	NR–47°C	23–50°C	25–52°C	10–50°C	25–50°C	25–52°C	28–42°C
Optimum temperature	37–42°C	40°C	38–40°C	30–45°C	35–40°C	45°C	36–45°C	37–45°C	37°C
Doubling time	1h	2h	NR	2.5h	2.9h	2.3h	40min	8h	NR
Carbohydrates utilized	+	+	–	+	+	+	+	Weak	–
End products of Fermentation	Acetate, Ethanol, H_2, CO_2	Acetate, Ethanol, H_2, CO_2	Acetate	Acetate, Isobutyrate, H_2, CO_2; TMA from Glycine betaine in the Stickland reaction	Acetate, Ethanol, H_2, CO_2	Acetate, Ethanol, Propionate, Formate, H_2, CO_2; TMA from Glycine betaine in the Stickland reaction	Acetate, Ethanol, Butyrate, Formate, H_2, CO_2	Acetate, Propionate, Isobutyrate, Iso-Valerate, H_2, CO_2	Acetate
Major fatty acids[1]	14:0 16:0 16:1	14:0 16:0 16:1	16:0 16:1	16:0 16:1	16:0 16:1	NR	14:0 16:0 16:1 18:0	16:0 16:1	NR
DNA mol% G+C	30.7	30.5	33.6	34.8	32.4	31.6	29.6	31.5	31.9
16S rRNA/rDNA accession number	X89074	NR	X89077	X89076	X89075	Y14212	X89073	M59122	X95817
Sample source	Sediment	Cyanobacterial mat	Sediment	Sediment	Silt	Sediment	Sediment	Sediment	Sediment
Site	Dead Sea	Lake Sivash, Crimea	Lake Sivash, Crimea	Saltern pond, California, USA	Lake Chokrak, Kerch Peninsula	Saltern pond, France	Dead Sea	Dead Sea	Lake Magadi, Kenya
Reference	Oren et al., 1984b	Zhilina et al., 1997	Zhilina and Zavarzin, 1990b	Liaw and Mah, 1992	Zhilina et al., 1992a, Rainey et al., 1995	Mouné et al., 1999	Oren et al., 1987; Rainey et al., 1995	Oren, 1983; Oren et al., 1987	Zhilina et al., 1996

L-BAPA hydrolysis, and three of them (*Halobacteroides halobius*, *Haloanaerobium praevalens*, *Orenia marismortui*) were found to hydrolyze D- but not L-BAPA. *Sporohalobacter lortetii* degraded neither of the BAPA stereoisomers (Oren et al., 1989).

Preservation

Several species of the *Haloanaerobiales*, notably *Halobacteroides halobius* (Oren et al., 1984b), *Orenia marismortui* (Oren et al., 1987), *Haloanaerobacter chitinovorans* (Liaw and Mah, 1992), *Haloanaerobacter lacunaris* (Zhilina et al., 1992a), *Haloanaerobacter salinarius* (Mouné et al., 1999), and *Natroniella acetigena* (Zhilina et al., 1996) easily undergo autolysis, during which spherical degeneration forms can be observed (see Figs. 1 B, C). Lysis starts at the end of the exponential growth phase, especially at relatively high growth temperatures. One possibility to avoid death of such cultures is the use of media with a reduced nutrient content and of lower growth temperatures (15–25°C). Weekly transfers may then suffice to maintain viable cultures.

The surest way to maintain cultures is by lyophilization, and so they are maintained by culture collections. *Haloanaerobium acetoethylicum* was successfully preserved by freezing anaerobic suspensions in 20% glycerol at –80°C (Rengpipat et al., 1988a).

Table 6. Media for the growth of members of selected members of the Haloanaerobiales (all values in g/liter, unless stated otherwise). Additional information can be found in the original species description papers at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (http://www.dsmz.de/media/media.htm).

	Halobacteroides halobius, Orenia marismortui	*Acetohalobium arabaticum*	*Haloanaerobacter chitinovorans*	*Sporohalobacter lortetii*	*Natroniella acetigena*
NaCl	140	150	100	105	15.7
$MgSO_4 \cdot 7H_2O$			9.6		
$MgCl_2 \cdot 6H_2O$	20.3	4.0	7.0	10[c]	0.1
KCl	3.7	0.33	3.8	0.75	0.2
NH_4Cl	7.35	0.33	1.0		1.0
$CaCl_2 \cdot 2H_2O$		0.33	0.5	3.7	
KH_2PO_4		0.33	0.4		0.2
K_2HPO_4					
$FeSO_4 \cdot 7H_2O$				2 mg	
$NaHCO_3$	5.0[a]	4.5[a]	3.0[a]		38.3[a]
Na_2CO_3			1.0[a]		68.3
$Na_2S \cdot 9H_2O$		0.5[a]	0.5[a]		1.0[a]
Glucose			5.0[a] or		
Chitin			5.0		
Microcrystalline cellulose or cellobiose					
Trimethylamine HCl or glycine betaine		2.4[a] or 4.5[a]			
Na-acetate					
Ethanol					5 ml[a]
Yeast extract	5.0	0.05[a]	1.0	2.0	0.2
Trypticase					
Peptone					
Casamino acids				2.0	
Nutrient broth				2.0	
L-glutamic acid				4.0	
Vitamin solution[b]		10 ml[a]		10 ml	10 ml
Trace element solution		10 ml[c]	1 ml[c]	10 ml[c]	1 ml[f]
Thioglycolate-ascorbate solution[g]					
Cysteine HCl			0.5[c]	0.5[c]	
Na-dithionite					
Resazurin	1 mg	1 mg	1 mg	1 mg	0.5 mg
NaOH 2N					
PIPES-di-K[h]	40 mM				
Final pH[i]	6.5–7.0	7.6–8.0	7.2	6.5	9.7–10.0

Table 6. *Continued*

	Halothermothrix orenii	*Halocella cellulolytica*
Former designation		
Type strain	OCM 544	DSM 7362
Cell size	$0.4–0.6 \times 10–20\,\mu m$	$0.4–0.6 \times 3.8–12\,\mu m$
Morphology	Rods	Rods
Motility	+	+
Flagella	Peritrichous	Peritrichous
Spores	–	–
Spheroplasts	NR	+
Gas vesicles	NR	NR
NaCl range	4–20%	5–20%
NaCl optimum	10%	15%
pH range	5.5–8.2	5.5–8.5
pH optimum	6.5–7.0	7.0
Temperature range	45–68°C	20–50°C
Optimum temperature	60°C	39°C
Doubling time	NR	NR
Carbohydrates utilized	+	+
End products of fermentation	Acetate, Ethanol, H_2, CO_2	Acetate, Ethanol, Lactate, H_2, CO_2
Major fatty acids[1]	14:0 $15:0_{iso}$ 16:0	14:0 16:0 $15:0_{anteiso}$
DNA mol% G+C	39.6	29.0
16S rRNA/rDNA accession number	L22016	X89072
Sample source	Sediment	Sediment
Site	Hypersaline lake, Tunisia	Lake Sivash, Crimea
Reference	Cayol et al., 1994b	Simankova et al., 1993

Physiology

All members of the Haloanaerobiales are strict anaerobes. They are oxidase and catalase-negative, and they do not contain cytochromes.

Most species grow fermentatively on sugars, producing acetate, ethanol, hydrogen, and carbon dioxide. Some strains produce in addition butyrate, lactate, propionate, and formate. *Haloanaerobium congolense* does not form ethanol (Ravot et al., 1997). The glucose transport system of certain Halobacteroides strains has been characterized in part (Senyushkin et al., 1992; Severina et al., 1992). Glycine betaine fermentation by *Haloanaerobium alcaliphilum* yields acetate and trimethylamine (Tsai et al., 1995). *Acetohalobium arabaticum* in pure culture accumulates little trimethylamine, as most is converted to acetate. In coculture with halophilic methanogenic bacteria, most trimethylamine formed is utilized by the methanogens (Zhilina and Zavarzin, 1990a).

Acetohalobium arabaticum and *Natroniella acetigena* have a homoacetogenic metabolism, producing acetate as the main end product of their energy metabolism. *Acetohalobium arabaticum* can grow on hydrogen + carbon dioxide or on carbon monoxide as a lithoautotroph, on trimethylamine as a methylotroph, and on other substrates (formate, trimethylamine, glycine betaine, lactate, pyruvate, histidine, aspartate, glutamate, and asparagine) as an organotroph (Kevbrin et al., 1995; Zhilina and Zavarzin,

1990a, 1990b). Electron transport from hydrogen during autotrophic growth is mediated by a flavoprotein, without involvement of NAD, cytochromes or quinones (Pusheva and Detkova, 1996; Pusheva et al., 1992). Activity of a Na^+/H^+ antiporter was shown to be important in the energy metabolism of this bacterium. Monensin, an inhibitor of Na^+/H^+ antiport and Na^+-dependent ATP synthesis, inhibited growth, and it was postulated that the Na^+ potential generated via Na^+/H^+ antiport is essential for the functioning of the cell (Pusheva and Detkova, 1996). The corrinoid metabolism of this organism has been studied as well (Bykhovsky et al., 1994).

All known members of the Haloanaerobiales can be classified as moderately halophilic. Most grow optimally at NaCl concentrations around 10–15%. A minimal NaCl concentration of 2–10% is required, depending on the species, and NaCl concentrations of up to saturation support growth in certain isolates. The most halophilic representative described thus far is *Haloanaerobium lacusrosei*, which grows between 6 and 34% NaCl with an optimum at 20% (Cayol et al., 1995).

Although belonging to the bacterial domain, the representatives of the order Haloanaerobiales display a number of physiological and biochemical properties characteristic of the halophilic Archaea rather than of the moderately halophilic aerobic Bacteria. Whereas the halophilic Bacteria (aerobes as well as anaerobic photosynthetic types) accumulate organic

osmotic solutes, such as glycine betaine and others, to balance the cytoplasm osmotically with the surrounding medium, the anaerobic halophilic Bacteria do not (Oren, 1986b; Oren et al., 1997; Rengpipat et al., 1988b). High concentrations of Na^+, K^+ and Cl^- were measured inside the cells of *Haloanaerobium praevalens*, *Halobacteroides halobius* (Oren, 1986b) and *Haloanaerobium acetoethylicum* (Rengpipat et al., 1988b), high enough to be at least isotonic with the medium. The intracellular ionic concentrations of individual cells of *Haloanaerobium praevalens* were assayed by means of X-ray microanalysis with the transmission electron microscope. Apparent intracellular cation concentrations between 1.22 and 1.91 M and chloride concentrations of 0.93–1.57 M were measured in cells growing exponentially in 2.6 M total salts. Because the cells flatten during sample preparation, these values probably underestimate the true intracellular concentrations. In exponentially growing cells, K^+ was the major cation (70% of the cation sum). Stationary phase cells showed a high variability among individual cells, part of the cells containing high concentrations of NaCl rather than KCl (Oren et al., 1997).

In accordance with the high intracellular salt concentrations, the intracellular enzymatic machinery should be adapted to function in the presence of high salt. Indeed, those enzymes tested (glyceraldehyde-3-phosphate dehydrogenase, NAD-linked alcohol dehydrogenase, pyruvate dehydrogenase, and methyl viologen-linked hydrogenase from *Haloanaerobium acetoethylicum*; hydrogenase and CO dehydrogenase activities of *Acetohalobium arabaticum*; and the fatty acid synthetase complex of *Haloanaerobium praevalens*) generally function better in the presence of molar concentrations of salts than in the absence of salts, and they can be expected to be fully active at the actual salt concentrations present in the cytoplasm (Oren and Gurevich, 1993; Rengpipat et al., 1988b; Zavarzin et al., 1994). Also similar to the aerobic halophilic Archaea, the tested members of the Haloanaerobiales have highly acidic bulk cellular protein (Oren, 1986b). However, no especially high content of acidic amino acids was found in the ribosomal A-protein of *Haloanaerobium praevalens* (Matheson et al., 1987).

Several strains (*Haloanaerobium saccharolyticum*, *Halobacteroides halobius*, *Halobacteroides elegans*, *Haloanaerobacter lacunaris*) can use methanethiol as the sole source of assimilatory sulfur for growth (Kevbrin and Zavarzin, 1992a; Zhilina et al., 1997). An interesting feature common at least to several members of the Haloanaerobiales is the ability to use oxidized sulfur compounds as electron acceptors or electron sinks. Thus, *Haloanaerobium congolense* uses thiosulfate and elemental sulfur as electron acceptors. Thiosulfate improved carbohydrate utilization and enhanced growth rates. Addition of thiosulfate or sulfur increased the growth yield 6-fold and 3-fold, respectively. In addition, the presence of thiosulfate alleviated growth inhibition by accumulating hydrogen (Ravot et al., 1997). Also, reduction of elemental sulfur to sulfide was reported in *Haloanaerobium saccharolyticum* (Zhilina et al., 1992b), in *Haloanaerobacter lacunaris* (Zhilina et al., 1992a), and in *Halobacteroides elegans* (Kevbrin and Zavarzin, 1992a; Zhilina et al., 1997). Moreover, thiosulfate reduction was observed in *Orenia marismortui* (Oren et al., 1987). *Acetohalobium arabaticum* was reported to slowly reduce sulfur to sulfide, but this was not accompanied by growth enhancement (Kevbrin and Zavarzin, 1992b; Zavarzin et al., 1994). Other compounds that can serve as electron sinks are nitro-substituted aromatic compounds such as nitrobenzene, nitrophenols, 2,4-dinitrophenol and 2,4-dinitroaniline, found to be reduced by *Haloanaerobium praevalens* and by *Orenia marismortui* (Oren et al., 1991, 1992).

Certain members of the Haloanaerobiales grow at high temperatures (*Halothermothrix orenii*) or at high pH (*Natroniella acetigena*, *Haloanaerobium alcaliphilum*). Little is known about the special adaptations of these bacteria that enable them to withstand both the salt stress and the additional stress caused by the high temperature or pH.

Ecology

Halophilic anaerobic Bacteria of the order Haloanaerobiales may be found in any hypersaline anaerobic environment in which simple organic compounds such as sugars and amino acids are available. They therefore abound in the anaerobic sediments of hypersaline lakes and saltern evaporation ponds, and they also appear to be widespread in anaerobic brines associated with oil deposits.

For the saccharolytic representatives, the ability to use substrates such as glycerol, glucosylglycerol, trehalose, cellulose and chitin may be of particular ecological importance. The first three compounds are accumulated at high concentrations as organic osmotic solutes by aerobic photosynthetic halophilic microorganisms inhabiting salt lakes (glycerol in the green unicellular alga *Dunaliella*, glucosylglycerol and trehalose in a variety of cyanobacteria). Thus, such compounds can be expected to be available to the anaerobic bacterial community in the bottom sediments of these lakes. *Haloanaerobium saccharolyticum* (both subsp. *saccharolyticum* and

subsp. *senegalense*) ferments glycerol (Cayol et al., 1994a; Zhilina et al., 1992b), and so does *Haloanaerobium lacusrosei* (Cayol et al., 1995). No details have been published on the fermentation products made when glycerol serves as energy source. Glycerol oxidation by anaerobic halophiles was reported to be markedly improved through interspecies hydrogen transfer when the glycerol fermenters were grown in coculture with H_2-consuming sulfate-reducing bacteria (Cayol et al., 1995). *Haloanaerobium saccharolyticum* was isolated from a cyanobacterial mat, dominated by *Microcoleus chthonoplastes* and covering the bottom of a hypersaline lagoon in the Crimea; its ability to use glucosylglycerol, the osmotic solute produced by *Microcoleus* and many other cyanobacteria, may be of great ecological importance (Zhilina and Zavarzin, 1991). The same organism also degrades trehalose, produced by other cyanobacteria for similar purposes (Zhilina et al., 1992b). The hypersaline lagoons of the Crimea also contain large masses of dead macroalgae (*Cladophora*). Such environments show high cellulolytic activity; the optimum salt concentration for cellulose decomposition was 15%, and decomposition was possible up to 25% salt. During cellulose decomposition, a transient accumulation of reducing sugars was observed in the medium (Simankova and Zavarzin, 1992). A cellulose-degrading halophilic anaerobe, *Halocella cellulolytica*, was isolated from this habitat (Simankova et al., 1993), and its cellulase complex was characterized in part (Bolobova et al., 1992). Another biopolymer that may be available in large quantities in hypersaline lakes is chitin, derived from the brine shrimp *Artemia* and from larvae of the brine fly, which are often abundant in such environments. Evolution of gas bubbles was observed from the sediment of a California saltern containing massive amounts of dead brine shrimp. Two strains of *Haloanaerobacter chitinovorans* were isolated from this saltern, of which only one grew on chitin. This strain was characterized by the presence of two chitin-induced extracellular proteins of 38 and 40 kDa molecular mass (Liaw and Mah, 1992). Another substrate that may be available abundantly in hypersaline environments is glycine betaine. This compound is produced as an osmotic solute by the most halophilic among the cyanobacteria and by halophilic anoxygenic photosynthetic bacteria such as *Halorhodospira* species. Glycine betaine was found to be fermented to acetate and trimethylamine by Haloanaerobium alcaliphilum isolated from the Great Salt Lake, Utah (Tsai et al., 1995), and by the (non-saccharolytic) *Acetohalobium arabaticum*. The latter species produces only minor amounts of trimethylamine, as most is converted to acetate (Zhilina and Zavar-

zin, 1990b). Glycine betaine can also be used as an electron acceptor in the Stickland reaction by *Haloanaerobacter salinarius* and *Haloanaerobacter chitinovorans*, with H_2 or serine as electron acceptor (Mouné et al., 1999).

Typical fermentation products of the saccharolytic representatives of the Haloanaerobiales are acetate, ethanol, hydrogen, and CO_2 (see Tables 2–3). The homoacetogenic *Acetohalobium arabaticum* can use hydrogen to reduce CO_2 for the production of additional acetate in a chemolithotrophic mode of life. Little is known on the fate of the other end products of the fermentation processes performed by the halophilic anaerobes in their natural habitats. The methylated amines formed during degradation of glycine betaine are excellent substrates for the growth of halophilic methanogenic bacteria (Zhilina and Zavarzin, 1990a) or for acetate production by *Acetohalobium arabaticum* (Zhilina and Zavarzin, 1990a, 1990b). No sulfate-reducing bacteria have been isolated as yet that grow at salt concentrations exceeding 20%, and no moderately or extremely halophilic methanogens are known that utilize hydrogen or acetate as energy sources. Acetate was suggested to be a dead-end product in the anaerobic halophilic community (Zhilina and Zavarzin, 1990a).

Halophilic anaerobic bacteria of the order Haloanaerobiales also have been found in environments, which in addition to the osmotic stress are characterized by extremes of temperature or pH. Thus, *Halothermothrix orenii*, the first truly thermophilic halophilic anaerobe, was isolated from Chott El Guettar, a warm saline lake in Tunisia. It grows optimally at 60°C, and up to 68°C at salt concentrations as high as 20%. As expected on the basis of its high temperature optimum, the isolate has unusual lipids, with branched-chain fatty acids (iso-15:0, anteiso-15:0, iso-17:0) accounting for about 75% of the fatty acids (Cayol et al., 1994b). An *Acetohalobium arabaticum* strain designated Z-7492, showing 78% DNA-DNA homology with the type strain, had a temperature optimum of 55°C (Kevbrin et al., 1995). Several halophilic anaerobes can grow at high pH values. *Haloanaerobium praevalens* from the Great Salt Lake was described as a moderate alkaliphile (Zeikus et al., 1983). *Haloanaerobium alcaliphilum*, isolated from the same environment, has a broad pH range (5.8–10) (Tsai et al., 1995). The alkaline (pH 10.2) Lake Magadi, Kenya, was shown to harbor a varied anaerobic community, including cellulolytic, proteolytic, saccharolytic, and homoacetogenic bacteria (Zhilina and Zavarzin, 1994). The homoacetogen *Natroniella acetigena* was isolated from this environment. This organism can grow on lactate, ethanol, pyruvate, glutamate, and propanol, forming acetate as the

main end product, and excreting propionate when grown on propanol. In contrast to *Acetohalobium arabaticum*, it is unable to grow autotrophically on hydrogen + carbon dioxide. Its pH optimum is 9.8–10.0, and growth has been observed up to pH 10.7 (Zhilina et al., 1996).

Quantitative data on the occurrence of members of the Halobacteriales in hypersaline anoxic environments are scarce. *Haloanaerobium praevalens* was reported to be present in Great Salt Lake surface sediment in numbers of up to 10^8 per ml sediment (Zeikus, 1983; Zeikus et al., 1983), where 10^3–10^5 *Halobacteroides halobius* cells were counted per ml of Dead Sea sediment (Oren et al., 1984b). Up to 10^7–10^9 anaerobic halophilic cellulolytic bacteria were enumerated per ml sediment in lagoons of the Arabat strait (Simankova and Zavarzin, 1992). Up to 4.6×10^3 anaerobic halophiles were counted in anaerobic brines associated with an oil reservoir in Oklahoma (Bhupathiraju et al., 1991, 1993). The few data available prove that these anaerobic halophiles may form a significant component of the ecosystem in anaerobic hypersaline sediments.

A search for novel organisms, both by using a wider variety of potential substrates and salt concentrations in enrichment cultures or in media for direct isolation and by extending the search to previously unexplored habitats, will undoubtedly yield a much greater variety of anaerobic fermentative bacteria than presently known.

Applications

The Haloanaerobiales have found few applications thus far. It has been suggested that they may be useful in microbially enhanced oil recovery from oil reservoirs by plugging of porous reservoirs and useful for producing commodities, such as gases, biosurfactants, polymers, etc., by anaerobically metabolizing nutrients in ambient environmental conditions (Bhupathiraju et al., 1991).

The use of anaerobic halophilic bacteria in the industrial fermentation of complex organic matter has been proposed, and preliminary tests have been made (Wise, 1987), but to my knowledge possible applications of the organisms in such processes are still in an experimental stage.

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CHAPTER 1.2.28

The Genus *Eubacterium* and Related Genera

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Phylogeny

The genus *Eubacterium*, because of its loose definition (see Taxonomy section below), includes species with a diverse range of phenotypes. Not surprisingly, therefore, 16S rRNA gene sequence comparison has revealed that the species of this genus are widely distributed within the phylogenetic tree. At present, 16S rRNA sequences have been determined for 45 of the 46 species and subspecies. These all fall within the phylum Firmicutes (Fig. 1). The type species, *Eubacterium limosum* belongs to a cluster with *Eubacterium callanderi* and *Eubacterium barkeri*, and it has been suggested that the genus *Eubacterium* sensu stricto should be restricted to these species (Willems and Collins, 1996), with the possible addition of *Eubacterium aggregans*, which also belongs to this phylogenetic group. Other major clusters of *Eubacterium* species within the Firmicutes include one consisting of *Eubacterium biforme*, *Eubacterium cylindroides*, *Eubacterium dolichum* and *Eubacterium tortuosum* within the family Erysipelotrichaceae, and a group of asaccharolytic species isolated from the oral cavity including *Eubacterium brachy*, *Eubacterium infirmum*, *Eubacterium minutum*, *Eubacterium nodatum*, *Eubacterium saphenum* and *Eubacterium sulci*, which are related to *Mogibacterium* (formerly *Eubacterium*) *timidum*. Other *Eubacterium* species are scattered among the Firmicutes and frequently have *Clostridium* species as close relatives. This is another heterogeneous genus of Gram-positive anaerobes; spore formation is the principal characteristic by which strains are placed within *Clostridium* rather than *Eubacterium*. The intermingling of species of these two genera throughout the phylogenetic tree suggests either that sporulation has emerged frequently throughout evolution or that it is a feature that has been lost from some species over time.

Other species recently transferred from *Eubacterium* such as *Eggerthella lenta* (Wade et al., 1999), *Collinsella aerofaciens* (Kageyama et al., 1999a), *Atopobium fossor* (Kageyama et al., 1999b), *Slackia exigua* (Wade et al., 1999) and the recently described genera *Cryptobacterium*

(Nakazawa et al., 1999) and *Olsenella* (Dewhirst et al., 2001) are found in the phylum Actinobacteria (Fig. 2), a phylum of Gram-positive organisms with DNA of mainly high G+C content.

Taxonomy

The genus definition for *Eubacterium* is rather unsatisfactory as it is defined by default; it includes those Gram-positive, nonsporeforming, obligately anaerobic rods that do not produce: 1) propionic acid as major acid (in contrast to *Propionibacterium*); 2) lactic acid alone (in contrast to *Lactobacillus*); 3) more acetic acid than lactic acid with and without formic acid (in contrast to *Bifidobacterium*); and 4) succinic acid (in the presence of carbon dioxide) and lactic acid with small amounts of acetic or formic acid (in contrast to *Actinomyces*; Moore and Holdeman Moore, 1986). Because of this loose definition, the genus is highly heterogeneous. The DNA G+C content of the current *Eubacterium* species is 30–57 mol%. As mentioned above, many of the species recently transferred to other existing or novel genera belong to the *Actinobacteria* and typically have high DNA G+C content, e.g., 62% for *E. lenta* and 60–64 % for *S. exigua*.

The majority of current species, therefore, do not belong in the genus *Eubacterium* sensu stricto and will be moved to other existing or novel genera in time. This process is under way and a number of species have been transferred already. In addition to those mentioned above, *E. formicigenerans* has been moved to the novel genus *Dorea* (Taras et al., 2002) and *E. timidum* is the type species of the new genus *Mogibacterium* (Nakazawa et al., 2000). *Eubacterium suis* has moved twice: first to *Actinomyces* (Ludwig et al., 1992) and then to *Actinobaculum* (Lawson et al., 1997).

Given the phylogenetic association between the genera *Eubacterium* and *Clostridium*, it is not surprising that many phenotypic features are shared. For example, most of the saccharolytic species of *Eubacterium* described form butyric acid and hydrogen gas, as do many saccharolytic
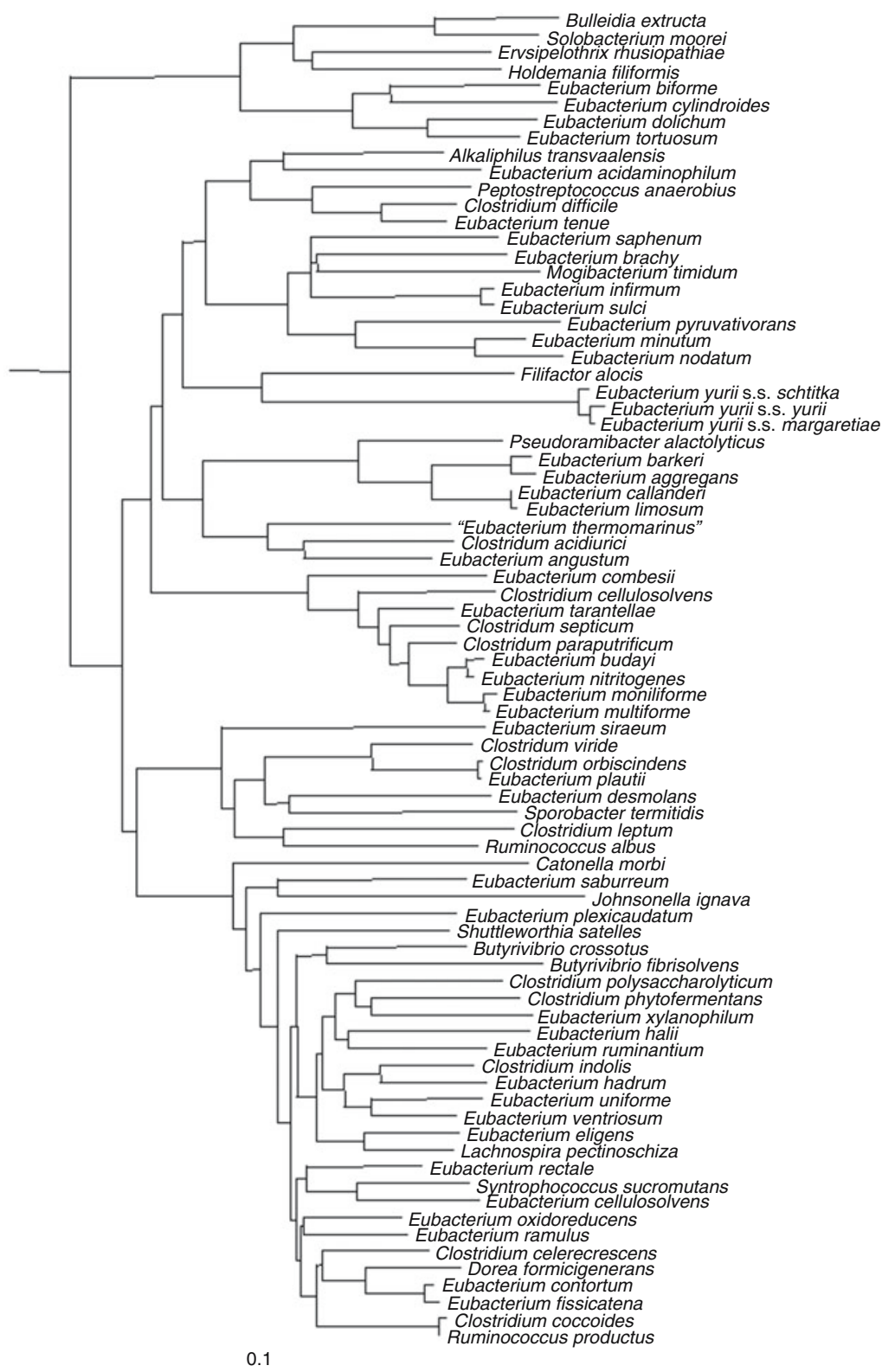
Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence comparisons showing *Eubacterium* species and related taxa within the phylum Firmicutes.
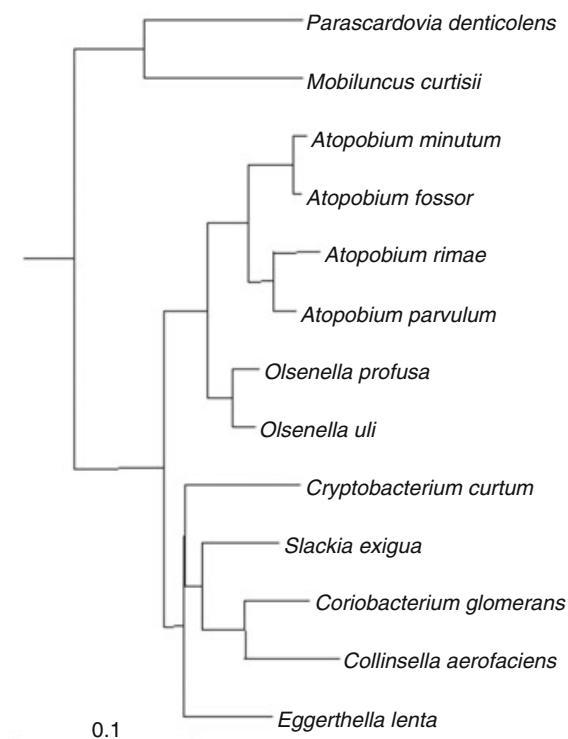
Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence comparisons showing *Eubacterium* species and related taxa within the phylum Actinobacteria.

Clostridium species. In addition to ethanol, butanol is formed by *E. saburreum* and *E. tenue*, as also seen in some solvent-producing clostridia (Bahl and Gottschalk, 1988). Caproic acid is generally quite seldom encountered as end product but can be a unique product of *Pseudoramibacter* (*Eubacterium*) *alactolyticus*, *E. biforme*, *E. limosum* and *E. pyruvativorans*, as well as of *Clostridium kluyveri*, *Peptococcus niger* and *Megasphaera elsdenii* (Holdeman et al., 1977; Moore and Holdeman Moore, 1986). Some *Eubacterium* spp. have the ability to degrade amino acids, as shown by the identification of isobutyric and isovaleric acids as products of *E. acidaminophilum*, *E. brachy*, *E. callanderi*, *E. combesii*, *E. limosum* and *E. tenue* (Moore and Holdeman-Moore, 1986; Mountfort et al., 1988; Zindel et al., 1988), and of phenylacetic acid as a product of *M. timidum* (Hill et al., 1987). As observed for clostridia (Andreesen et al., 1989), specialists able to degrade purines (*E. angustum*), trihydroxybenzenoids (*E. oxidoreducens*), O-methoxylated aromatic acids (*E. callanderi*) or steroids (*E. desmolans*) have also been isolated (Beuscher and Andreesen, 1984; Krumholz and Bryant, 1986; Morris et al., 1986; Mountfort et al., 1988). Cholesterol reduction is a particular feature of *E. coprostanoligenes* (Freier et al., 1994). Autotrophic growth on H_2 plus CO_2 or on one-

carbon compounds such as CO, formate and methanol is also possible in *E. limosum* (Genthner et al., 1981; Genthner and Bryant, 1982; Genthner and Bryant, 1987).

Some *Eubacterium* species (for instance *E. plautii*) are either Gram-negative (Hofstad and Aasjord, 1982) with very occasional weak Gram-positive areas in the cells or they decolorize rather easily e.g., *E. cylindroides*. The ambiguity of this fundamental character has caused considerable confusion, for example, *Eubacterium sulci* was originally considered to be Gram-negative and as such was originally described as a member of the genus *Fusobacterium* (Cato et al., 1985; Jalava and Eerola, 1999). Therefore, these species can only be classified with certainty by demonstrating the absence of lipopolysaccharides, especially of 3-hydroxy fatty acids, and by analysis of the outer membrane proteins (Baardsen et al., 1988). The chemical composition of the peptidoglycan has been proven to be a useful taxonomic marker since members of this group display a wide variety of types, such as the *meso*-diaminopimelic acid-direct type (Alγ according to the nomenclature of Schleifer and Kandler, 1972) in *E. acidaminophilum*, *E. angustum*, *E. alactolyticum*, *E. saburreum* and *E. tenue*; the LL-diaminopimelic acid-glycine type (A3γ) in *E. combesii* and *E. lenta*; various unique structures in *E. nodatum* and *A. suis*; and the B2α type (found in *E. limosum* and *E. callanderi*) where peptide bridges are connected to the D-glutamic acid residue (Tanner et al., 1981; Weiss, 1981; Wegienek and Reddy, 1982; Beuscher and Andreesen, 1984; Zindel et al., 1988; Severin et al., 1989a; Severin et al., 1989b). *Holdemania filiformis*, phylogenetically related to the genus *Erysipelothrix*, resembles it in having type B cell wall murein. Indeed, further analysis revealed the murein to be of a type previously undescribed: B1δ(l-Ala)-D-Glu-Gly-L-Lys (Willems et al., 1997). Whether the related species *Bulleidia extructa* and *Solobacterium moorei* possess a similarly unusual murein composition remains to be determined.

Habitat

Eubacterium species are found in the mammalian mouth and intestinal tract (including the rumen) and in the environment (Table 1).

In quantitative studies of the human intestinal flora, *Eubacterium* species were found to be the second most numerous genus with a median count of about 6×10^{10} organisms per g of dry weight of feces compared with 10^{11} cells per g of *Bacteroides* species (Finegold et al., 1983). *Eubacterium* species were recovered from 94% of subjects and *C. aerofaciens*, *E. contortum*, *E.*

Table 1. Habitats of species of *Eubacterium* and related genera.

Species	Human infections	Human mouth	Human gut	Mammalian gut	Rumen	Environment	Other
E. limosum	•		•	•	•	•	
E. acidaminophilum						•	
E. aggregans						•	
E. angustum						•	
E. barkeri			•			•	
E. biforme			•				
E. brachy	•	•	•				
E. budayi			•			•	
E. callanderi						•	
E. cellulosolvens					•		
E. combesii	•					•	
E. contortum	•		•				
E. coprostanoligenes			•	•		•	
E. cylindroides			•	•			
E. desmolans			•	•			
E. dolichum			•				
E. eligens			•	•			
E. fissicatena							
E. hadrum			•				
E. hallii			•				
E. infirmum	•	•					
E. minutum	•	•					
E. moniliforme	•		•			•	
E. multiforme	•					•	
E. nitritogenes	•					•	
E. nodatum	•	•					
E. oxidoreducens					•		
E. plautii							Cultures of *Entamoeba histolytica*

(Continued)

Table 1. *Continued*

Species	Human infections	Human mouth	Human gut	Mammalian gut	Rumen	Environment	Other
E. plexicaudatum				•			
E. pyruvativorans					•		
E. ramulus			•		•		
E. rectale			•				
E. ruminantium					•		
E. saburreum	•	•					
E. saphenum	•	•					
E. siraeum			•				
E. sulci	•	•	•				
E. tarantellae							Brains of dead or moribund striped mullet
E. tenue	•						
E. tortuosum			•	•		•	Turkey liver granulomas
E. uniforme					•		
E. ventriosum	•		•	•			
E. xylanophilum	•	•			•		
E. yurii subsp. *yurii*	•	•					
E. yurii subsp. *margaretiae*	•	•					
E. yurii subsp. *schtitka*	•	•					
Bulleidia extructa	•	•					
Collinsella aerofaciens	•		•				
Eggerthella lenta	•		•				
Holdemania filiformis			•				
Shuttlworthia satelles	•	•					
Slackia exigua	•	•					
Solobacterium moorei	•	•	•				

cylindroides, *E. lenta* and *E. rectale* were identified in at least 20% of the cases, demonstrating these to be the predominant *Eubacterium* spp. in that habitat. *Collinsella aerofaciens* was present in 40–60% of the cases, independent of the diet or state of health, whereas the presence of other species varied from three- to sixfold. Subsequent studies of the intestinal flora have resulted in the description of many new species, including *E. dolichum*, *E. eligens*, *E. formicigenerans*, *E. hadrum*, *E. hallii*, *E. ramulus* and *E. siraeum* (Holdeman and Moore, 1974; Holdeman et al., 1976; Moore et al., 1976). *Eubacterium ramulus* is of particular interest as it is capable of degrading a wide range of flavonoids, compounds obtained from food and thought to be beneficial to health (Simmering et al., 1999). Schwiertz et al. (2000) used oligonucleotide probes to detect ten *Eubacterium* species in human fecal samples. *Eubacterium biforme*, *E. cylindroides*, *E. hadrum*, *E. ventriosum* and *E. lenta* were detected, and overall the results were similar to those previously seen in cultural studies.

The majority of *Eubacterium* species found in the human mouth are asaccharolytic and mainly found in oral disease. The predominant species are *E. brachy*, *E. nodatum*, *E. saphenum*, *M. timidum* and *S. exigua* (Holdeman et al., 1980; Uematsu and Hoshino, 1992; Uematsu et al., 1993; Poco et al., 1996). Some saccharolytic species are found, and these include *E. saburreum*, *E. yurii*, *B. extructa* and *S. satelles*. *Eubacterium yurii* forms interesting cellular arrangements; all three subspecies form three-dimensional "test tube brush" arrangements from "*Actinomyces*-like" granules, which are perpendicular to a central stalk (Margaret and Krywolap, 1986; Margaret and Krywolap, 1988).

Many of the species found in the environment are also found in infectious material. This suggests that *Eubacterium* species naturally occurring in soil, for example, can act as opportunist pathogens in man.

Some species appear to be associated with specific host organisms. For example, *E. tarantellae* has been isolated from infected fish brains (Udey et al., 1977), *Atopobium fossor* from the pharynx and tooth abscesses of horses (Bailey et al., 1986), and *E. plautii* as an endosymbiont of *Entamoeba histolytica* (Hofstad and Aasjord, 1982).

The rumen is an ideal habitat for *Eubacterium* species because of its rich supply of organic materials (Stewart and Bryant, 1988). *Eubacterium ruminantium* seems to be well adapted to these conditions for it shows a requirement for sodium and rumen fluid and constitutes about 3–7% of the total isolates using a nonspecific medium (Bryant, 1959). *Eubacterum limosum* is one of the nutritionally most versatile species, degrading carbohydrates, amino acids, lactate and methanol, and even growing autotrophically on $H_2 + CO_2$ or CO (Genthner et al., 1981; Genthner and Bryant, 1982). Strains of *E. limosum* have been isolated from the rumen fluid of sheep but not of a steer. The fiber-degrading species *E. cellulosolvens*, *E. uniforme* and *E. xylanophilum* can be specifically enriched using cellulose, cellobiose or xylan as the selective substrate (Van Gylswyk and Van der Toorn, 1985; Van Gylswyk and Van der Toorn, 1986). Xylan utilizers represent about 6% of the total viable count, with *E. uniforme* as the more versatile organism present (at about 5%) and *E. xylanophilum* as a specialist for cellobiose and xylan (at about 1%) of the xylan-degrading flora (Van Gylswyk and Van der Toorn, 1985). Lignins, tannins and flavonoids have trihydroxybenzenoids, so that trihydroxybenzenoids are common constituents of the rumen diet. *Eubacterium oxidoreducens* degrades gallate, pyrogallol, phloroglucinol and quercetin in the presence of formate or hydrogen as electron donors; crotonate can be degraded without an exogenous electron donor. *Eubacterium* oxidoreducens cannot grow on conventional media in the absence of one of these substrates (Krumholz and Bryant, 1986). A pathway for gallate and phloroglucinol degradation to acetate and butyrate has been proposed (Krumholz et al., 1987), and the enzymes catalyzing unique reactions, like pyrogallol-phloroglucinol isomerase and phloroglucinol reductase, have been characterized (Krumholz and Bryant, 1988; Haddock and Ferry, 1989). *Eubacterium pyruvativorans* is found in the sheep rumen and does not ferment sugars but can grow on pyruvate or trypticase, forming caproate as the major endproduct (Wallace et al., 2003).

Man-made environments for the treatment of waste of various sorts have also been shown to be habitats capable of supporting the growth of *Eubacterium* species. *Eubacterium limosum* and *E. callanderi* have been isolated from the anaerobic digesters of sewage plants (Genthner et al., 1981; Mountfort et al., 1988). *Eubacterium callanderi* is somewhat specialized for the cleavage of phenylether bonds present in O-methoxylated aromatic substrates such as ferulate, sinapate, syringate, vanillate or 3,4,5-trimethoxycinnamate (Mountfort et al., 1988). *Eubacterium angustum*, isolated from sewage sludge, has a restricted substrate range, utilizing only few purine compounds like uric acid, xanthine, guanine and hypoxanthine (Beuscher and Andreesen, 1984). Purines form a major fraction of the cell constituents and are excreted as N-rich compounds by birds, reptiles, insects and other animals (Vogels and Van der Drift, 1976). *Eubacterium acidaminophilum* is another nutritionally modest organism isolated from sludge, utilizing in pure culture only glycine and serine

as single substrates. However, its substrate spectrum is extended to other amino acids or to some organic acids if a hydrogen-accepting organism or an acceptor like betaine, creatine or sarcosine is also present (Zindel et al., 1988; Hormann and Andreesen, 1989). *Eubacterium coprostanoligenes*, isolated from a hog sewage lagoon, reduces cholesterol to coprostanol but does not require it for growth, although lecithin is needed (Freier et al., 1994). *Eubacterium aggregans* was isolated from a particularly specialized site: an anaerobic digestor fed with wastewater from an olive mill (Mechichi et al., 1998).

Isolation

For the majority of species, the complex media recommended for anaerobes by the Anaerobe Laboratory of the Virginia Polytechnic Institute (Holdeman et al., 1977) and the Wadsworth-KTL Anaerobe Laboratory Manual (Jousimies-Somer et al., 2002) are adequate for growth. However, because of the still-unresolved nutritional requirements of some species of *Eubacterium*, these media have to be supplemented with complex ingredients (vitamins, rumen fluid, volatile fatty acids, and substrates specific to the species) to obtain reasonable growth. Although these rich media appear to contain all necessary nutrients, growth of some species is still poor (Hill et al., 1987). To enhance growth of *E. acidaminophilum*, for example, selenium as a special micronutrient and a hydrogen acceptor or donor must be present (Zindel et al., 1988). If these are present in a balanced ratio, good growth will even occur in a simple defined medium. A salt solution (containing $CaCl_2$, $MgCl_2$, potassium phosphate, $NaHCO_3$ and NaCl; Holdeman et al., 1977) is a constituent of most media used to cultivate *Eubacterium* species. However, some species are inhibited by phosphate concentrations higher than 5 mM where the pH-buffering capacity is quite low (Zindel et al., 1988).

Most of the *Eubacterium* species are highly sensitive towards oxygen. Only *E. desmolans* can tolerate 4 h of exposure to atmospheric air (Morris et al., 1986). Nitrate reduction is a property quite seldom encountered within the genus *Eubacterium*, especially as a marker valid for all strains (Bokkenheuser et al., 1979; Moore and Holdeman-Moore, 1986). This might indicate that most of the species within the genus are true anaerobes.

Identification

The species described in this chapter are difficult to identify. Many are slow-growing, have fastidi-

ous nutritional requirements, and are unreactive in the commonly used biochemical and physiological tests. For these reasons, particular caution should be exercised using commercially available rapid identification kits. The indifferent growth of many *Eubacterium* species can make the constituent tests in these kits especially unreliable and furthermore the preponderance of negative reactions makes interpretation of the test results difficult. If identification by phenotypic means is attempted, the test methods described by Holdeman et al. (1977) are recommended. As mentioned before, the assignment of an organism to the genus *Eubacterium* depends on the nature of its metabolic products, their ratio on certain standard media, and on the pH decrease observed during the fermentation of carbohydrates to the respective acids (Moore and Holdeman-Moore, 1986). Fermentation tests should be performed with pre-reduced anaerobically sterilized sugars as long as growth in the basal medium is adequate. Only by using identical, well-specified conditions, can the species be differentiated by identification keys and tables (Moore and Holdeman-Moore, 1986). Production (or absence) of butyric acid and acid formation leading to a sharp pH drop (or none) are the main lines of differentiation. Selected characteristics used for preliminary identification of *Eubacterium* and related species are given in Table 2. Certain species of *Eubacterium* cannot be definitively differentiated; for instance, *E. budayi* and *E. nitritogenes* are very closely related in their phenotypes. Furthermore, if *Eubacterium* spp. also hydrolyze gelatin, they could be mistaken for *Clostridium perfringens*, a situation that might also obtain for *E. moniliforme* and *E. ventriosum* (Moore and Holdeman-Moore, 1986).

Increasingly, 16S rRNA sequence analysis is becoming the method of choice for bacterial identification, and this method works well for this group. The 16S rRNA sequences of the majority of *Eubacterium* species show sufficient sequence divergence from their close neighbors for identification to be possible, with a partial sequence of around 500 base pairs. An exception however is the genus *Mogibacterium*, where the species *M. diversum* and *M. neglectum* cannot be easily distinguished from *M. vescum* by 16S rRNA analysis (Nakazawa et al., 2002).

The use of 16S rRNA gene sequence analysis for identification of clinical isolates provisionally identified as "*Eubacterium*" has revealed that a significant proportion do not correspond to named species (Downes et al., 2001; Munson et al., 2002). Although progress is being made in the proposal of new genera and species to accommodate these strains, much remains to be done and it can be expected that numerous novel taxa

Table 2. Principal biochemical characteristics of *Eubacterium* species and related taxa.

Selected characteristics	Utilization of sugars (restricted to few)	Utilization of amino acids or formation of branched chain fatty acids	Formation of — Copious H₂	Butyrate	Caproate	Ethanol	Butanol	Formate	Lactate	Stereoisomer of lactate	Nitrate reduction	G+C content (mol%)	Variable in Gram stain
E. acidaminophilum	−	+	−	−	−	(+)	−	−	−	None	−	44	(+)
E. aggregans	+	n.r.	(+)	+	−	n.r.	n.r.	+	+	None	n.r.	55	−
E. angustum	−	−	−	+	−	+	+	+	+	None	−	40	−
E. barkeri	+	n.r.	(+)	+	−	n.r.	n.r.	−	+	nd	n.r.	45	−
E. biforme	+	−	+	+	(+)	−	−	+	+	nd	n.r.	32	−
E. brachy	−	+	−	−	−	−	+	(+)	(+)	nd	−	39	+
E. budayi	+	−	+	+	−	−	−	−	−	(L)	+	n.r.	+
E. callanderi	+	+	(+)	+	−	−	−	+	(+)	nd	+	47	nd
E. cellulosolvens	+	−	(+)	−	−	−	−	(+)	+	(D)	n.r.	n.r.	−
E. combesii	−	+	+	+	−	(+)	(+)	(+)	+	nd	−	n.r.	−
E. contortum	+	−	+	+	−	+	+	+	−	None	−	45	−
E. coprostanoligenes	+	−	−	−	−	−	−	+	−	None	−	41	−
E. cylindroides	+	−	−	+	−	−	−	(+)	−	(DL)	−	31	+
E. desmolans	−	−	+	+	−	−	−	(+)	(+)	nd	−	35	−
E. dolichum	−	−	+	+	−	−	−	(+)	(+)	nd	n.r.	n.r.	−
E. eligens	+	−	−	−	−	+	−	+	+	nd	+	36	+
E. fissicatena	+	−	+	+	−	+	−	(+)	(+)	nd	−	46	−
E. hadrum	+	−	+	+	−	−	−	+	+	nd	+	32–33	−
E. hallii	+	−	+	+	−	−	+	(+)	(+)	nd	(+)	36–38	−
E. infirmum	−	+	n.r.	+	−	n.r.	n.r.	−	−	None	n.r.	38	+
E. limosum	+	+	+	+	+	−	−	−	−	None	−	46–48	−
E. minutum	−	+	n.r.	+	−	n.r.	n.r.	−	−	None	−	38–40	−
E. moniliforme	+	−	+	+	−	−	(+)	+	+	(L/DL)	(+)	n.r.	−
E. multiforme	+	−	+	+	−	−	(+)	+	+	None	+	n.r.	+
E. nitritogenes	+	−	+	+	−	−	(+)	+	+	(DL)	+	n.r.	+
E. nodatum	−	+	−	+	−	+	+	(+)	(+)	−	nd	36–38	−
E. oxidoreducens	−	−	−	+	−	+	−	−	−	None	−	36	−

Selected characteristics	Utilization of sugars (restricted to few)	Utilization of amino acids or formation of branched chain fatty acids	Formation of — Copious H₂	Butyrate	Caproate	Ethanol	Butanol	Formate	Lactate	Stereoisomer of lactate	Nitrate reduction	G+C content (mol%)	Variable in Gram stain
E. plautii	+	−	+	+	−	−	−	+	+	nd	n.r.	n.r.	+
E. plexicaudatum	+	−	+	−	+	−	+	−	−	None	n.r.	44	−
E. pyruvativorans	−	+	n.r.	−	+	n.r.	n.r.	−	(+)	None	n.r.	57	−
E. ramulus	−	−	+	+	−	−	−	+	+	nd	n.r.	39	−
E. rectale	+	+	+	+	−	−	−	+	+	nd	−	30	−
E. ruminantium	+	−	−	−	−	−	−	+	+	(DL)	(+)	n.r.	+
E. saburreum	+	−	+	+	−	+	+	(+)	+	nd	−	41	+
E. saphenum	−	+	n.r.	+	−	n.r.	n.r.	−	−	None	n.r.	44–48	−
E. siraeum	+	+	+	+	−	+	−	(+)	nd	nd	−	45	−
E. sulci	−	+	−	−	−	n.r.	n.r.	−	−	None	n.r.	39	+
E. tarantellae	+	+	+	+	−	−	−	(+)	nd	−	n.r.	−	
E. tenue	+	+	+	+	−	−	−	+	+	None	n.r.	−	
E. tortuosum	+	−	(+)	+	−	−	−	+	+	(DL)	(+)	n.r.	−
E. uniforme	+	−	−	−	−	+	+	+	+	(L)	−	35	−
E. ventriosum	+	−	−	+	−	−	−	+	+	(D)	n.r.	−	
E. xylanophilum	(+)	−	−	+	−	−	−	+	−	None	−	39	−
E. yurii	(+)	−	−	−	−	−	−	−	None	32	−		
A. fossor	(+)	−	−	−	−	−	−	+	nd	43–46	−		
A. suis	+	−	−	−	+	−	+	(+)	nd	55	−		
B. extructa	+	+	n.r.	−	n.r.	n.r.	+	n.r.	38	−			
C. aerofaciens	+	−	+	+	(+)	−	+	(D/DL)	60	−			
C. curtum	−	+	n.r.	−	n.r.	−	−	None	50–51	+			
D. formicigenerans	−	−	(+)	+	+	+	nd	40	−				
E. lenta	−	+(Arg)	−	−	−	(+)	(+)	nd	+	62	−		
M. timidum	−	(+)(Phe)	−	−	−	(+)	(+)	nd	50	+			
P. alactolyticus	+	−	+	+	+	−	+	−	None	61	−		
S. moorei	+	n.r.	−	+	−	n.r.	−	+	nd	37–39	+		
S. zatelles	+	n.r.	n.r.	+	−	n.r.	n.r.	+	nd	51	−		

Symbols and abbreviations: +, present; −, absent; (+) nd, no data.

belonging to this group will be described in the years to come.

Disease

The genus *Eubacterium* was proposed by Prévot (1938) to describe a group of bacteria isolated from human feces that were considered to be beneficial. Although no *Eubacterium* species have been shown to be capable of causing infections alone, a number of species are isolated from infections (Table 1), with the primary source of the infecting organisms thought to be the lower gut. *Eubacterium lenta* is the species most frequently isolated.

Some species have been implicated as key players in the multispecies biofilms associated with infections in the mouth. For example, Uematsu and Hoshino (1992) showed that *Eubacterium* constituted 54% of the microflora in periodontal pockets, and Moore et al. (Moore et al., 1982; Moore et al., 1983) showed that *E. brachy*, *E. nodatum*, *M. timidum* and *S. exigua* were associated with periodontally diseased sites. Serum antibodies to these organisms were found in periodontal patients compared to controls, providing further evidence of their active involvement in the disease process (Tew et al., 1985; Gunsolley et al., 1990; Smith and Wade, 1999). *Eubacterium* species are also frequently isolated from other oral infections including dental caries (Hoshino, 1985) and endodontic (Munson et al., 2002) and dentoalveolar infections (Wade et al., 1993).

Eubacterium species of oral origin have also been reported to cause opportunistic infections at a variety of other body sites (Allen, 1985; Hill et al., 1987; Finegold and George, 1989). Reports of other *Eubacterium* species causing human disease include *E. plautii* causing a fulminant infection following a dog bite (Garre et al., 1991) and a case of bacteremia caused by *E. callanderi* (Thiolas et al., 2003). *Collinsella aerofaciens* has been implicated in rheumatoid arthritis (Eerola et al., 1994) and the particular chemical composition of its cell wall peptidoglycan appears to be responsible for its arthritogenicity (Zhang et al., 2001).

Applications

As emphasized above, species of *Eubacterium* and related genera, especially *E. lenta*, represent a dominant group of organisms in the human gut. Although this species is normally inactive in most biochemical tests, organisms phenotypically related to it can catalyze a wide variety of transformation reactions on steroids (Bokken-

heuser et al., 1979; MacDonald et al., 1983; Edenharder and Mielek, 1984; Hylemon, 1985; Akao et al., 1988) or are able to split off the side chain of cortisol, as is characteristic for *E. desmolans* (Bokkenheuser et al., 1986; Morris et al., 1986). So far, biotransformations of steroids carried out by anaerobes have not received any industrial attention (Sedlaczek, 1988), although the inability of these organisms to cleave the ring system of steroids might be regarded as an advantage.

Some *Eubacterium* species can reduce cholesterol to coprostanol. Most of these isolates require the presence of alkenyl ether lipids (plasmalogens) as a specific growth factor (Mott and Brinkley, 1979). *Eubacterium coprostanoligenes* reduces cholesterol via the formation of 4-cholesten-3-one, which is then reduced to coprostanol with coprastanone as an intermediate (Ren et al., 1996). The possibility of using this organism in a probiotic way to reduce blood cholesterol levels has been investigated in hens and mice, but the results to date have been equivocal (Li et al., 1996; Li et al., 1998).

Cortisol desmolase and 20-β-hydroxysteroid dehydrogenase are unique for *E. desmolans* (Bokkenheuser et al., 1986; Morris et al., 1986). A 21-dehydroxylating activity leading from 11-deoxycorticosterone (21-hydroxy-4-pregnene-3, 20-dione) to progesterone is known for *E. lenta* (Bokkenheuser et al., 1977). This species can also produce a 16 α-dehydroxylase (Bokkenheuser and Winter, 1980), a 12 α- or 12 β-hydroxysteroid dehydrogenase (MacDonald et al., 1983), and bile acid 3 α-, 3 β-, 7 α-, and 12 α-hydroxysteroid dehydrogenase (Edenharder and Mielek, 1984), and forms ω-muricholic acid from the β-derivative (Eyssen et al., 1983). Epimerization reactions occur via the corresponding keto-compounds and might require the cometabolism of two species (MacDonald and Hutchinson, 1982; Edenharder and Schneider, 1985; Canzi et al., 1989).

The 7-dehydroxylase activity of *Eubacterium* sp. VPI 12 708 (closely related to *E. lenta*) seems to form a novel bile acid nucleotide to labilize the 7-hydroxy group to generate a 3-keto-Δ4,6-steroid nucleotide intermediate (Coleman et al., 1987). Proteins and even genes involved in the 7-dehydroxylation reaction have been characterized (Coleman et al., 1987; White et al., 1988a). A multigene family seems to be involved, and a 27-kDa protein exhibits extensive sequence homologies to alcohol and polyol dehydrogenases (White et al., 1988b).

The activity of the enzyme 16-hydroxyprogesterone dehydroxylase in combination with a 16-dehydroxyprogesterone reductase and progesterone reductase to form finally 17-isopregnanedione are 10-fold higher if H_2 or pyruvate is

present (Glass and Burley, 1985). A study of many strains similar to *E. lenta* demonstrated that a few strains were also able to deconjugate both glycine and taurine conjugates of cholic acid and chenodeoxycholic acid. All strains studied could reduce one double bond of linoleic acid to *trans*-vaccenic acid (Eyssen and Verhulst, 1984). The reduction of the unsaturated lactone ring of digoxin, a cardiac glycoside, results in the formation of 99% pure 20R-dihydrodigoxin without hydrolysis of the glycosidic bond by *E. lenta* (Robertson et al., 1986). Also, the digitoxin derivatives are selectively reduced to the 20R-form during active growth (Chandrasekaran et al., 1987). Thus, many stereoselective biotransformation reactions can be performed by a variety of strains of *E. lenta*.

Eubacterium limosum is the other species that is of interest to biotechnologists because of its production of cobamides (Perlman and Semar, 1963; Vogt et al., 1988) and carboxylic acids (Bryant and Genthner, 1983; Lindley et al., 1987). The organism is a part of several highly efficient methanogenic consortia, because of its high metabolic versatility (Zellner and Winter, 1987).

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CHAPTER 1.2.29

The Genus *Mycoplasma* and Related Genera (Class Mollicutes)

SHMUEL RAZIN

Introduction

Mycoplasmas are distinguished phenotypically from other bacteria by their minute size and total lack of a cell wall. Taxonomically, the lack of cell walls is used to separate mycoplasmas from other bacteria and to place them in a class named "Mollicutes" (*mollis*, soft; *cutis*, skin, in Latin). The classification of Mollicutes and the properties distinguishing the established taxa are presented in Table 1. Though the trivial terms mycoplasmas or mollicutes have been used interchangeably to denote any species included in Mollicutes, the trivial names "ureaplasmas," "entomoplasmas," "mesoplasmas," "spiroplasmas," "acholeplasmas," "asteroleplasmas," and "anaeroplasmas" are routinely used for members of the corresponding genus. Molecular characterization of the uncultured plant and insect mycoplasma-like organisms (MLOs) has provided strong experimental support for their inclusion in the class Mollicutes (see the section Phylogeny and Taxonomy). Consequently, the trivial term "phytoplasmas" has been proposed to replace the awkward name "mycoplasma-like organisms." About 200 established species have already been described within the class Mollicutes, and this number continues to rise, including the still uncultured mollicutes, such as the phytoplasmas and the hemotrophic parasites *Haemobartonella* and *Eperythrozoon* species, recently identified as mollicutes and given the trivial name "hemoplasmas" (Neimark et al. 2001).

The interested reader is referred to a number of books on various aspects of mycoplasmology published during the last decade or so (Maniloff et al., 1992; Rottem and Kahane, 1993; Razin and Tully, 1995; Tully and Razin, 1996; Miles and Nicholas, 1998). The most recent multi-authored treatise covering the molecular biology and pathogenicity of mycoplasmas has been published in the summer of 2002 (Razin and Herrmann, 2002). Several recent general reviews on the molecular biology, genetics and pathogenicity of mycoplasmas are also available (Dybvig and Voelker, 1996; Baseman and Tully, 1997; Razin et al., 1998).

Morphology

The total lack of a cell wall explains many of the unique properties of the mycoplasmas, such as sensitivity to osmotic shock and detergents, resistance to penicillin, and formation of peculiar fried-egg-shaped colonies (Razin and Oliver, 1961; Fig. 1). Thin sections of mycoplasmas reveal that the cells are built essentially of three organelles: the cell membrane, ribosomes, and a circular double-stranded DNA molecule (the typical prokaryotic genome; Fig. 2). Because they are devoid of a cell wall, mycoplasmas are Gram negative. Being bounded by a plastic cell membrane only, mycoplasma cells are predominantly spherical (0.3–0.8μm in diameter). Yet, many can be pear-shaped or flask-shaped with terminal tip structures, and form filaments of varying length (some branching) as well as helical filaments (Figs. 3–5). Maintaining such shapes in the absence of a rigid cell wall has indicated long ago the presence of a cytoskeleton in mycoplasmas (Razin, 1978).

Motility and Cytoskeletal Elements

Most mycoplasmas are nonmotile and have no flagella. However, some of the flask-shaped *Mycoplasma* species, including the human and animal pathogens (*M. pneumoniae*, *M. genitalium*, *M. gallisepticum*, *M. pulmonis* and *M. mobile*) glide on liquid-covered surfaces. *Mycoplasma mobile*, isolated from fish gills, is the fastest (2.0–4.5μm/sec). The mechanism of this peculiar gliding motility is still unknown (Trachtenberg, 1998; Miyata, 2002). Although *M. pneumoniae* is motile and exhibits chemotactic behavior, gliding motility genes could not be identified in this mycoplasma, as it is not yet known which genes to look for. Furthermore, none of the components of the chemotactic signal pathway (the Che proteins, which are well conserved among bacteria) or any other two-

Table 1. Major characteristics and taxonomy of the class Mollicutes.

Classification	No. of recognized species	Genome size (kb)	Genome G+C (mol%)	Cholesterol requirement	Distinctive properties	Habitat
Mycoplasmataceae						
Genus I: *Mycoplasma*	107 (11)[a]	580–1350	23–40	Yes	Optimum growth 37°C	Humans and animals
Genus II: *Ureaplasma*	7	760–1170	27–30	Yes	Urease positive	Humans and animals
Entomoplasmataceae						
Genus I: *Entomoplasma*	6	790–1140	27–29	Yes	Optimum growth 30°C	Insects and plants
Genus II: *Mesoplasma*	12	870–1100	27–30	No	Optimum growth 30°C	Insects and plants
Spiroplasmataceae						
Genus I: *Spiroplasma*	34	780–2220	24–31	Yes	Helical filaments	Insects and plants
Acholeplasmataceae						
Genus I: *Acholeplasma*	14	1500–1650	26–36	No	Optimum growth 30–37°C	Animals and plant surfaces
Anaeroplasmataceae						
Genus I: *Anaeroplasma*	4	1500–1600	29–34	Yes	Obligate anaerobes, oxygen sensitive	Bovine–ovine rumen
Genus II: *Asteroleplasma*	1	1500	40	No		
Undefined taxonomic status						
Phytoplasma	ND[b]	530–1185	23–29	ND	Uncultured in vitro	Insects and plants

Abbreviation: ND, not determined.
[a]The number of Candidatus species is given in parentheses and includes the hemoplasmas (*Eperythrozoon* and *Haemobartonella*) recently transferred to the genus *Mycoplasma* (Neimark et al., 2001).
[b]The taxonomic status of the uncultured phytoplasmas has not been finally defined; seven Candidatus *Phytoplasma* spp. have so far been published.
Updated and modified from Razin et al. (1998).
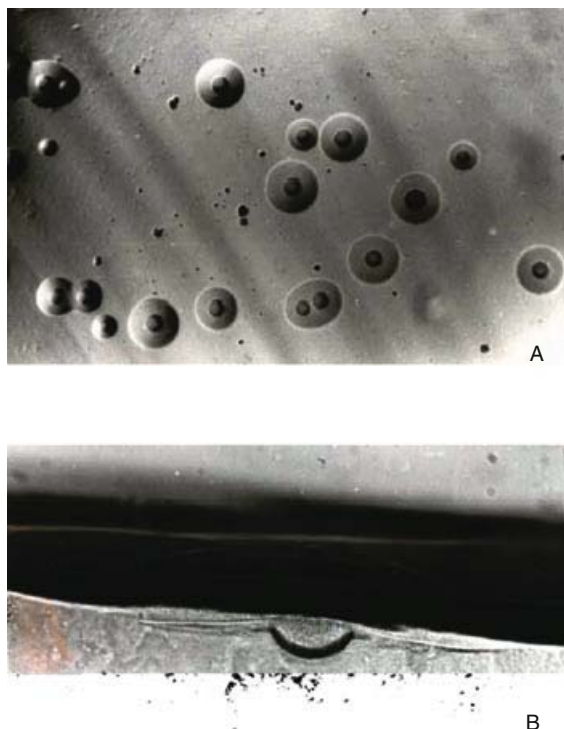
Fig. 1. *Acholeplasma laidlawii* colonies on mycoplasma agar, magnification 50X. A) View from above shows the characteristic "fried-egg" shape consisting of a central zone embedded in the agar and a peripheral zone on the agar surface. B) Vertical section of a mycoplasma colony shows the central zone embedded in the agar and the peripheral zone located in the free water film on the agar surface. From Razin and Oliver (1961).

Fig. 3. Scanning electron micrograph of A) *Mycoplasma gallisepticum* cells showing the polymorphic shape of the organisms. B) *Mycoplasma pneumoniae* showing the characteristic elongated cells with tapered tips as well as spherical cells and microcolonies. Bar = 1µm. From Razin et al. (1980).

Fig. 2. Electron micrograph of thin-sectioned mycoplasma cells. The cells are bounded by a single membrane exhibiting the characteristic trilaminar shape of sectioned biomembranes fixed in osmium tetroxide. The cytoplasm contains thin threads representing the sectioned chromosome, and dark granules representing ribosomes. Courtesy of R. M. Cole, Bethesda, MD.

component signal transduction system could be identified in the sequenced *M. pneumoniae* and *M. genitalium* genomes (Himmelreich et al., 1996). The ability of the gliding mycoplasmas to cytadhere mirrors closely their ability to bind to glass, a prerequisite for gliding. Because cytadherence is linked to the tip structure (see below), this attachment organelle probably plays a major role in the gliding mechanism. In fact, the gliding mycoplasmas always move with the attachment organelle at the leading end (Miyata, 2002).

Spiroplasma species are unique in having helical morphology (Fig. 5), rotary motility, and chemotaxis. Motility is characterized by a rapid spinning along the long axis of the cell as well as
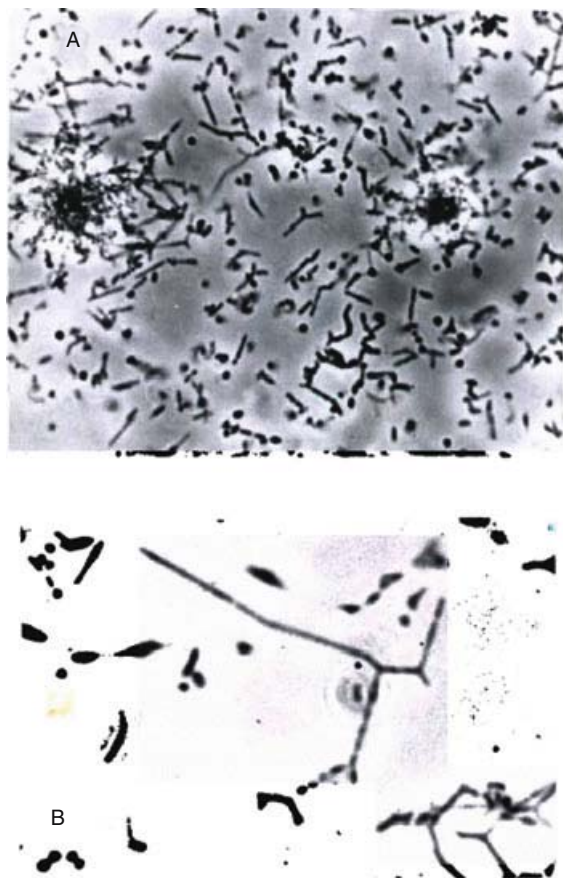
Fig. 4. Phase-contrast light microscopy of *Acholeplasma laidlawii* cells. A) Magnification 1250×. (B) Magnification 2700×. Branching filaments and chains of cocci can be seen in these unfixed live broth cultures. From Razin et al. (1966).

Fig. 5. Helical filaments of *S. citri* negatively stained with 2% ammonium molybdate.

by flexional or undulating movements of the helical filaments. The spiroplasma cell can essentially be viewed as a membranal tube (diameter about 0.25 μm) to which a flat cytoskeletal ribbon made of 7 parallel fibrils (diameter 45–50 Å) is attached to its inner side. Both tube and ribbon are mutually coiled into a dynamic helix. The ribbon follows the shortest (inner) helical path (Trachtenberg, 1998). This can be regarded as an internal cytoskeleton that functions as a linear motor as well (Trachtenberg and Gilad, 2001; Trachtenberg et al., 2003a). Upon cell lysis by deoxycholate, spiroplasmas release the fibrils, which consist of a 56-kDa protein. The gene for this protein (*fib*) has been cloned, facilitating the characterization of the structural features of the fibril protein (Williamson et al., 1991). Some information on a gene involved in *S. citri* motility has been obtained through transposon mutagenesis (Jacob et al., 1997; see the section Gene Transfer). An *S. citri* nonmotile mutant was generated by Tn*4001* transposon mutagenesis. The transposon was inserted into a gene named

"*scm1*," encoding a putative polypeptide of about 45 kDa with no significant homology to any known protein. The *scm1* gene was recovered from the wild motile strain and inserted into an *S. citri* cloning vector. Transfection of the nonmotile mutant with this recombinant plasmid restored motility, indicating that the *scm1* gene product is indeed involved in *S. citri* motility, probably serving as an anchor of the cytoskeletal ribbon to the membrane (Trachtenberg and Gilad, 2001). Interestingly, the nonmotile mutant multiplied in the insect vector and was efficiently transmitted to plants, inducing disease and suggesting that spiroplasma motility may not be essential for pathogenicity (Duret et al., 1999).

Williamson et al. (1991) have suggested that an actin-like protein found in spiroplasmas may be linked to the fibrils and associated with motility. The presence of actin-related proteins in prokaryotes in general, and in mollicutes in particular, has long been suspected (Razin, 1978). The complete genomic analyses of *M. genitalium* and *M. pneumoniae* (Fraser et al., 1995; Himmelreich et al., 1996) failed to identify actin-related gene(s), leaving this issue unresolved. Thus far, significant sequence homologies between eukaryotic cytoskeletal proteins and any bacterial protein have not been found (Proft et al., 1995; Mayer et al., 1998), though Wasinger et al. (1995), applying the proteome technology, have suggested the presence of a tubulin-like protein in *M. genitalium*. Recent data, reviewed in Errington (2003) and by Carballido-Lopez and Errington (2003), indicate that actin-like proteins in bacteria form a cytoskeleton that helps to determine cell shape, much as happens in eukaryotic cells. The most studied actin-like proteins are
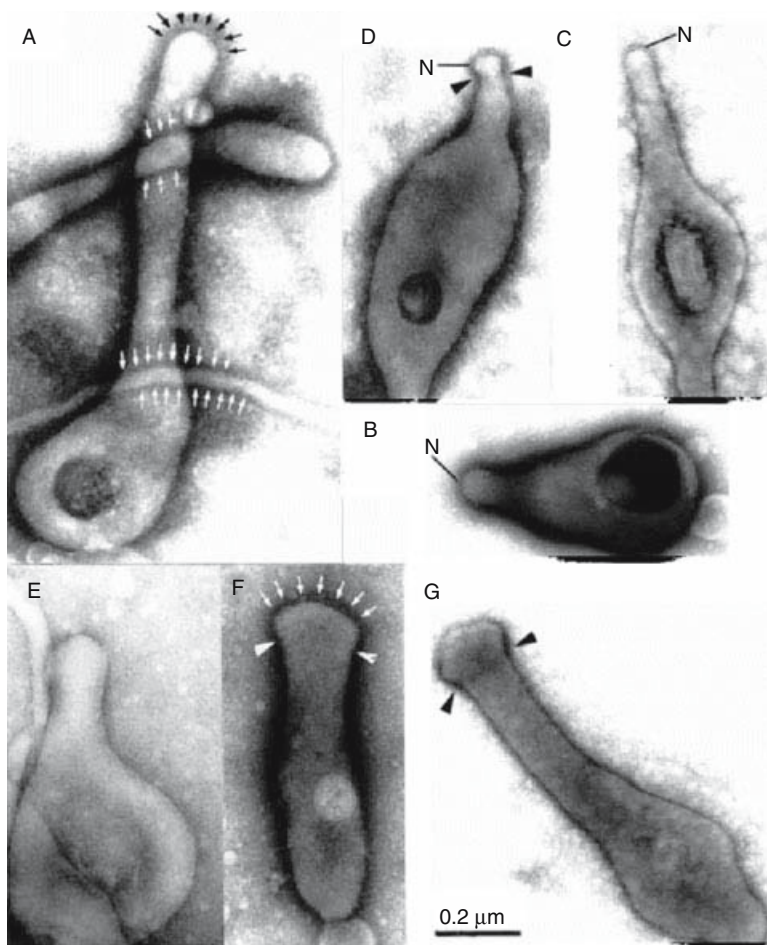
Fig. 6. Electron micrographs of negatively stained mycoplasmas possessing tip structures. A) *Mycoplasma pulmonis*. Arrows show a thin surface layer on top of a protruding stalk. B) *Mycoplasma gallisepticum*, showing the distinct bleb structure. The particulate surface nap (N) is not restricted to the bleb structure. C) *Mycoplasma pneumoniae*. Note the tapered terminal structure covered by the nap (N). D) *Mycoplasma genitalium*. Arrowheads show the tip structure covered by a nap (N) consisting of small peplomer-like particles. E) *Mycoplasma alvi* with a broadened, blunt, terminal structure covered by a thin amorphous surface layer (arrows). G) *Mycoplasma mobile*. Note the broadened head-like structure (arrowheads), with no surface layer. All photos from Kirchhoff et al. (1984).

those encoded by the *mreB* genes that are conserved widely in eubacteria, although they are not ubiquitous. Thus, no *mreB* genes were found in the *M. genitalium* and *M. pneumoniae* genomes. Possibly, mycoplasmas have related proteins forming filamentous structures resembling the Mbl proteins in *Bacillus subtilis* (Errington, 2003), a point awaiting further study.

An important group of pathogenic mycoplasmas, including *M. pneumoniae* and *M. genitalium*, shares a flask- or club-like cell shape with a protruding tip or bleb structure (Fig. 6). These mycoplasmas attach to eukaryotic cells via the tip structure (the mycoplasma's attachment organelle; Fig. 7; see the section Adhesion to Host Cells). Scanning- and transmission electron microscopy of *M. pneumoniae* cells grown on grids treated with 1% Triton X-100 revealed a rod-like tip structure and a network of filamentous strands (Meng and Pfister, 1980). Proteins of this so-called "Triton shell" are apparently
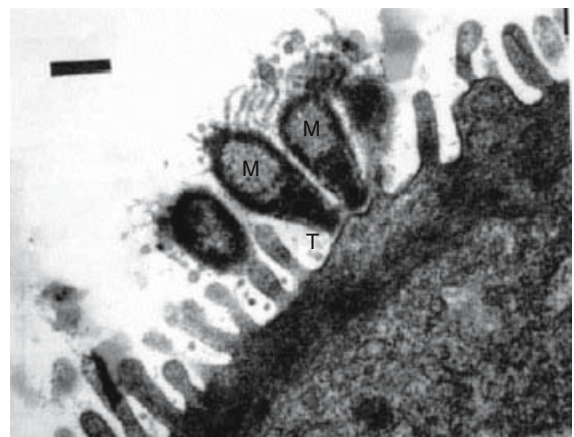
Fig. 7. Thin sections of *Mycoplasma gallisepticum* attaching to tracheal epithelium of chicken seven days postinfection. The mycoplasmas can be seen to attach through their blebs. M = mycoplasma and T = attachment tip. Bar = 0.5μm. From Razin (1985) and reprinted with the courtesy of M. J. Dykstra and S. Levisohn.

Fig. 8. Transformation of a filament to a chain of cocci. Cinematographic pictures of a *Mycoplasma hominis* culture grown in a cover slip chamber under the phase-contrast microscope. Minutes lapsed between pictures taken of (a) and (b) 2.8; (c) 3.3; (d) 4.8; (e) 5.3; or (f) 5.5. From Bredt et al. (1973).
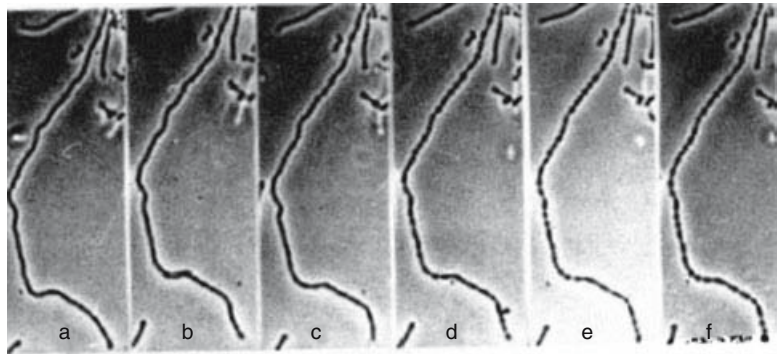
cytoskeleton-forming or cytoskeleton-associated proteins. The cytoskeleton-like structure is thought to function in modulating cell shape and to participate in cell division, gliding motility, and the proper localization of adhesins on the mycoplasmal cell surface.

The genomic analysis of *M. pneumoniae* (Himmelreich et al., 1996) has enabled the identification and molecular characterization of major protein building blocks of the cytoskeleton of this mycoplasma. Some of these proteins function as surface-exposed adhesins, including proteins P1 and P30, whereas others, named accessory proteins (designated "HMW1," "HMW2" and "HMW3" and "A," "B" and "C") collectively maintain the proper distribution and disposition of the adhesins in the mycoplasma membrane (Layh-Schmitt and Herrmann, 1994; Layh-Schmitt et al., 1995; Hahn et al., 1998). Additional proteins, named "P65" and "P200" (Proft et al., 1995, 1996) share characteristic structural features with HMW1 and HMW3, suggesting their function as elements of the *M. pneumoniae* cytoskeleton consistent with their presumed scaffolding role (Krause and Balish, 2001; Balish and Krause, 2002).

Recent analysis of the *M. pneumoniae* Triton shell by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry revealed about 100 protein spots. Regula et al. (2001) identified 41 of them. The identified proteins belonged to several functional categories, mainly energy metabolism, translation, and heat-shock response. In addition, lipoproteins and most of the proteins involved in cytadherence were found in this fraction (see the section Adhesion to Host Cells). Quantitatively, the most prevalent proteins were the heat-shock protein DnaK, elongation factor Tu, and subunits α and β of the pyruvate dehydrogenase complex (Regula et al., 2001). Thus, the Triton shell consists of a very heterogeneous complex of proteins as could perhaps be expected from a complex cell fraction isolated according to its insolubility at a low concentration of the mild detergent Triton X-100.
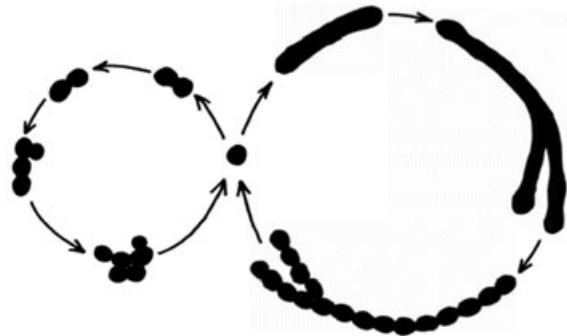
Fig. 9. Schematic presentation of the mode of mycoplasma reproduction. Cells may either divide by binary fission or elongate first to multinucleate filaments and subsequently break up to coccoid bodies. From Razin (1981).

Cell Division

The mode of reproduction of mycoplasmas is essentially no different from that of othe prokaryotes dividing by binary fission. For typical binary fission to occur, cytoplasmic division must be fully synchronized with genome replication, and in mycoplasmas, cytoplasmic division may lag behind genome replication, resulting in the formation of multinucleate filaments. When followed by microcinematography, the transformation of mycoplasma filaments into chains of cocci (a process taking a few minutes) was marked by the appearance of constrictions in the cell membrane at about equal distances along the entire length of the filament (Figs. 8–10). The recent sequencing of several mycoplasma genomes has provided some information on mycoplasmal genes homologous to cell division genes of wall-covered bacteria. The most important finding, perhaps, is that of the *ftsZ* gene in mycoplasmas (Bork et al., 1995; Fraser et al., 1995; Himmelreich et al., 1996; Wang and Lutkenhaus, 1996; Kukekova et al., 1999). In eubacteria, FtsZ is a polymer-forming, guanosine triphosphate (GTP)-hydrolyzing protein with tubulin-like elements; it is localized to the

a
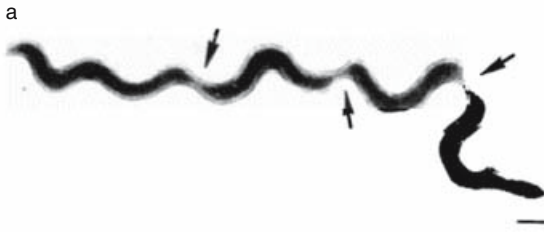
Fig. 10. Constrictions in the negatively stained helical filament of *Spiroplasma citri* preceding its division. Bar = 500 nm. From Razin (1978) reprinted with the courtesy of I. M. Lee.

site of septation and forms a constricting ring (the Z ring) between the dividing cells. The finding of *ftsZ* in mollicutes indicates that it is a highly conserved and ubiquitous gene fulfilling a key role in prokaryote cell division. Yet, generalizations are risky as FtsZ could not be found in *Ureaplasma* (Glass et al., 2000) and *Chlamydia* (Galperin, 2001). Of the additional genes associated with cell division in eubacteria (*ftsA*, *ftsH*, *ftsI*, *ftsQ*, *ftsW* and *ftsY*), *ftsH* was identified in *M. pneumoniae* (Himmelreich et al., 1996) and *M. capricolum* (Bork et al., 1995), and *ftsY* in *M. genitalium* (Fraser et al., 1995), *M. pneumoniae* (Himmelreich et al., 1996) and *U. urealyticum* (Glass et al., 2000). More recently, the *ftsY* of *M. mycoides* subsp. *mycoides* was cloned and characterized, and its role in protein targeting by complexing with the signal-recognition particle (SRP) was investigated (Macao et al., 1997). Applying the proteome approach, Wasinger et al. (1995) proposed the presence of a tubulin-like protein in *M. genitalium*. It has been suggested that the FtsZ protein of prokaryotes is a tubulin homologue, but the tubular structures formed by FtsZ in vitro are substantially different from microtubules, and the similarity between FtsZ and tubulin sequences is barely significant (Gupta and Soltys, 1996). Recent data (reviewed in Garballido-Lopez and Errington [2003]) have shed new light on the identity of the components of the bacterial cytoskeleton. Apparently, the bacterial FtsZ can be considered as the bacterial ancestor of tubulin, and the prokaryotic protein MreB is the bacterial equivalent of actin. Despite a weak sequence similarity (explaining the failure to identify these proteins as tubulin and actin in prokaryotic genomic annotations), FtsZ and MreB have structural, biochemical and functional properties strikingly similar to those of their eukaryotic counterparts. FtsZ undergoes tubulin-like GTP-dependent polymerization into a ring structure that constricts at septation and direct cytokinesis in almost all bacteria examined. MreB proteins undergo actin-like

ATP-dependent polymerization into helical structures that control morphogenesis in nonspherical bacteria (Garballido-Lopez and Errington, 2003).

An integral part of cell division is chromosome replication and partition into daughter cells. Chromosome replication and partitioning in mollicutes follows the basic principles of binary fission (Miyata and Seto, 1999; Miyata, 2002). The velocity of the DNA replication fork progression in *M. capricolum* was about 6kb/min, about 10 times slower than that of *E. coli*. The time required for one round of chromosome replication was estimated to be about 94 min, and *M. capricolum* is considered among the fastest growing mollicutes (Seto and Miyata, 1998). While the outline of DNA replication in mycoplasmas is presumably similar to that of walled bacteria, the coupling of chromosome replication and cell division in mycoplasmas is not so simple. The lack of a peptidoglycan layer, which plays important roles in nucleoid partitioning and cell division, argues that the concepts developed for walled bacteria cannot be simply applied to mycoplasmas (Seto and Miyata, 1999). Nucleoid movement occurs in combination with cell elongation, but the kind of force that propels and localizes the nucleoids is not known. Mycoplasma cells apparently lack a solid structure like the periseptal annulus in *E. coli*, because they do not have the peptidoglycan layer. Possibly, the fibrillar cytoskeletal structure observed in *M. pneumoniae* and *M. gallisepticum* is involved in nucleoid migration (Seto and Miyata, 1999).

Mycoplasmas carry topoisomerases (see the section DNA Replication and Repair) capable of relaxing superhelical DNA, fulfilling an essential role in cell division (Bailey et al., 1996). In mycoplasma species possessing a tip structure, duplication of this organelle is a prerequisite for cell division. Observation of the cell division process in *M. pneumoniae* suggests that the nascent organelle is formed next to the old one and then migrates to the opposite cell pole before binary fission of the cell takes place (Seto et al., 2001). What is the motive force propelling the migration of the tip organelle? This is certainly an intriguing question for which no answer is as yet available. One possibility is to associate it with the gliding motility of this mycoplasma. Another possibility is to associate it with the filamentous structures taking part in the cell division process (Seto et al., 2001). In conclusion, the factors coordinating the cell division process in mollicutes are still rather far from being clearly understood, inasmuch as genetic information has just started to accumulate and is still fragmentary and not entirely analyzed and evaluated.

Phylogeny and Taxonomy

Phylogeny

The early studies of Woese et al. (1980), comparing 16S rRNA sequences of mollicutes have provided the basis for mycoplasma phylogeny. Accordingly, the mycoplasmas arose at a node on the branch of Gram-positive bacteria having a low G+C genome. This branch of the *Lactobacillus* group contains *Lactobacillus*, *Bacillus* and *Streptococcus* species, and two rather unusual *Clostridium* species, *C. innocuum* and *C. ramosum*. Mycoplasma phylogeny from this node took place by degenerative or reductive evolution, characterized by a rapid tempo, in line with the marked genotypic and phenotypic variability characterizing the mollicutes as a group (Woese et al., 1985; Woese, 1987). Phylogenetic trees of Mollicutes are still based mostly on 16S rRNA sequences (Fig. 11), but several other conserved gene sequences (e.g., *tuf* genes) have been used for this purpose (Kamla et al., 1996). According to 16S rRNA sequences, the mollicutes are divided into five phylogenetic units (clades): the asteroleplasma, spiroplasma, pneumonia, hominis, and anaeroplasma clades (Weisburg et al., 1989; Johansson and Petersson, 2002).

A scheme of mycoplasma evolution has been proposed by Maniloff (2002). Accordingly, the ancestral mycoplasma arose from the *Streptococcus* phylogenetic branch about 600 million years ago, having probably a genome of about 2000 kb. The mycoplasma branch split later into two major branches: one branch led to the *Asteroleplasma*, *Anaeroplasma* and *Acholeplasma* branches, and the other led to the *Spiroplasma*, *Entomoplasma* and *Mycoplasma* branches. The phytoplasmas subsequently arose from the *Acholeplasma* branch, and *Ureaplasma* arose from the *Mycoplasma* branch. In both major branches, genome reductions had occurred independently during their degenerate evolution. Thus, degenerate evolution of the *Acholeplasma-Anaeroplasma* branch, taking place after the appearance of flowering plants, has led to the phytoplasmas with 600–1200-kb genomes, while evolution of the second major branch produced the *Spiroplasma* branch with 1000–2000-kb genomes and the *Entomoplasma*, *Mesoplasma*, *Mycoplasma* and *Ureaplasma* branches with 600–1200-kb genomes. Clearly, the time at which transition to rapid evolution occurred correlate with major paleontological events in the evolution of the flora and fauna that formed ecosystems for lineages of each branch. Thus, the *Spiroplasma* and *Entomoplasma* transition to rapid evolution occurred about 100 million years ago soon after the appearance of the first flowering plants and their associated insects. The

increase in potential hosts presumably provided niches for the selection of new lineages. Although all the above should be taken as hypothetical assessments, they emphasize a cardinal point: the mollicutes are relatively late evolutionary products.

The voluminous data on the molecular properties of mycoplasmas derived from the recent genomic analyses have provided strong support for the close phylogenetic relatedness of mollicutes to Gram-positive bacteria. Thus, in the majority of cases where *M. genitalium* coding regions matched sequences of both *Escherichia coli* and *Bacillus* species, the better match was to sequences of *Bacillus* (average, 62% similarity) than to sequences of *E. coli* (average, 56% similarity; Fraser et al., 1995). Other findings supporting this thesis are brought up in the Genetics and Physiology sections of this chapter.

Taxonomy

There is a consensus among bacterial taxonomists that the complete sequences of bacterial genomes will form the basis for phylogeny and, ultimately, taxonomy. Yet, as long as complete genomic sequences are available for only a relatively small number of bacteria, current bacterial taxonomy, including that of mollicutes, must rely on the combination of phenotypic characteristics and phylogenetic data based on partial genomic sequences, mostly those of the conserved ribosomal RNA genes. Detailed descriptions and evaluations of current molecular methods in taxonomy, phylogeny and diagnostics of mollicutes can be found in Razin (1992), in the two volumes of *Molecular and Diagnostic Procedures in Mycoplasmology* (Razin and Tully, 1995; Tully and Razin, 1996) and in *Mycoplasma Protocols* (Miles and Nicholas, 1998).

Though the availability of complete genomic sequences is expected to provide a sound basis for establishing phylogenetic relatedness among bacterial species and consequently enable the construction of taxonomic entities based on phylogeny, the way to achieve this has not been worked out as yet because of several key problems. Thus, the current, somewhat arbitrary definition of the basic taxonomic entity (i.e., the bacterial species) includes all strains with DNA having approximately 70% or higher homology and a melting temperature change (δT_m) of 5°C or lower (Razin, 1992b). There can be little doubt that this definition will have to be modified once comparison of entire genomic sequences replaces the cumbersome and not too accurate determination of genetic homology based on DNA-DNA hybridization tests. Yet, as long as the number of bacterial genomes sequenced remains small, there is still more than enough
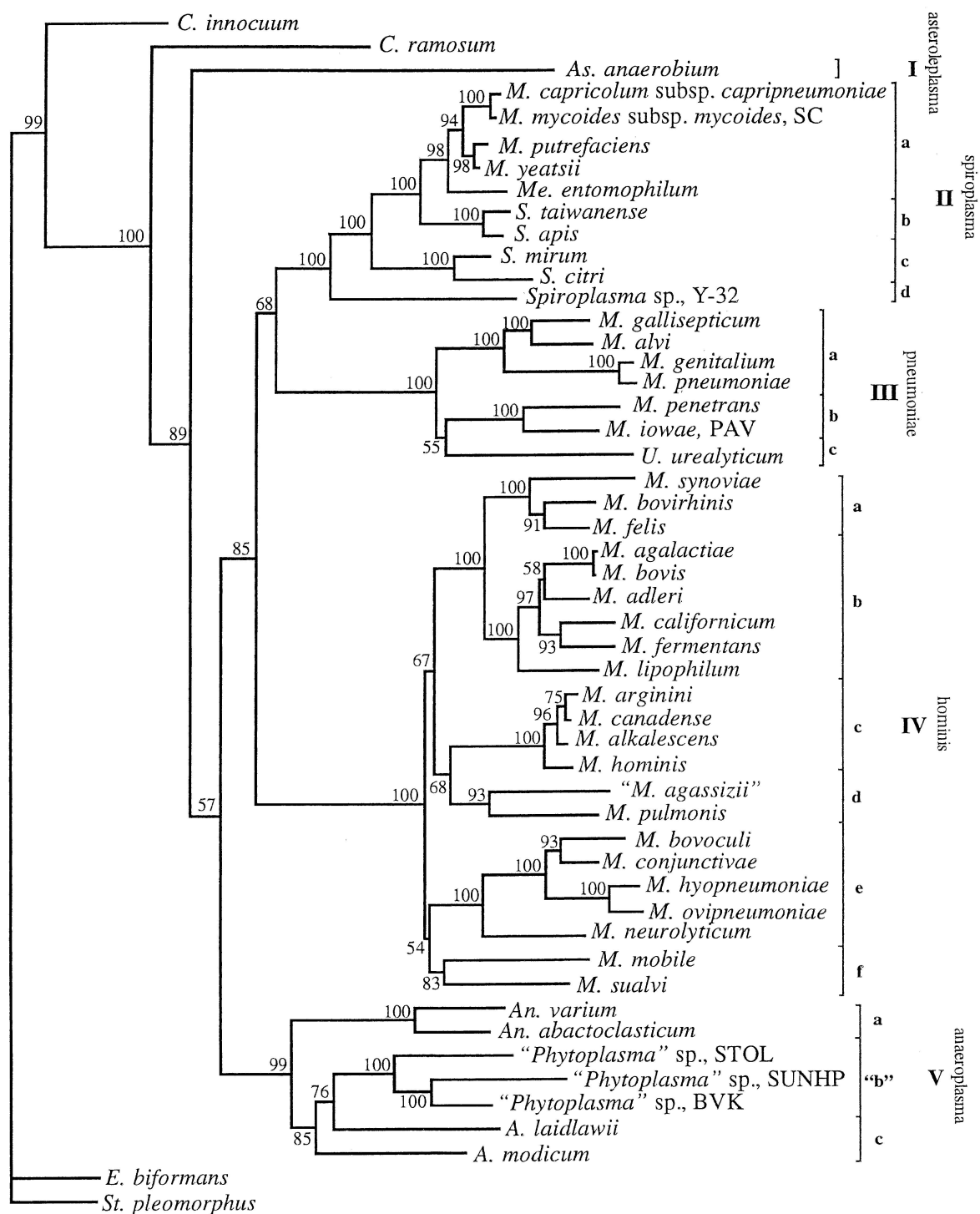
Fig. 11. Phylogenetic tree of mycoplasmas based on 16S rRNA sequences. Representatives of the closely related genera *Clostridium* and *Eubacterium* are also included in the tree. From Johansson et al. (1998).

time to work out a solution to the above problem. Comparison of the genomic sequences of *M. genitalium* and *M. pneumoniae* (Himmelreich et al., 1997; Herrmann and Reiner, 1998) has revealed the significant genomic relatedness of these mycoplasmas and underlines the problems that taxonomists may face when using comparative genomic data to redefine taxonomic entities, including that of a bacterial species.

Conserved Genes as Molecular Markers

The great weight given to 16S rDNA sequences in mycoplasma phylogeny, taxonomy and species identification (Razin, 1992; Johansson and Petersson, 2002) has led the International Committee on Systematic Bacteriology—Subcommittee on the Taxonomy of Mollicutes (1997)—to recommend the inclusion of the 16S rDNA sequence in any description of a new mollicute species. K.-E. Johansson has been compiling a database of 16S rDNA sequences of the recognized *Mycoplasma* species deposited at GenBank (Johansson and Petersson, 2002).

A most important contribution of 16S rRNA gene sequences to mollicute phylogeny and taxonomy has been the placing of the uncultured phytoplasmas (as a distinct monophyletic clade) within Mollicutes, closely related to the acholeplasmas (Lee et al., 2000; Seemuller et al., 2002). This conclusion has been supported by the small genome size of phytoplasmas, resembling that of the culturable mollicutes (Marcone et al., 1999), by the finding of only two rRNA operons in the phytoplasmas, and a tRNA[Ile] gene in the spacer region between the 16S and 23S rRNA genes (Ho et al., 2001; Seemuller et al., 2002), a feature found also in acholeplasmas. The deduced amino acid sequences of some of the highly conserved ribosomal protein genes also indicated that the phytoplasmas are more closely related to *Acholeplasma laidlawii* than to *Mycoplasma* species. Like acholeplasmas, phytoplasmas read UGA as a stop codon rather than a tryptophan codon. The polymerase chain reaction (PCR)-amplified 16S rRNA gene sequences delineated (within the phytoplasma clade) 20 major phylogenetic groups or subclades. This number is generally in accord with the phytoplasma strain clusters established by DNA homology, restriction enzyme analyses (REA) of PCR-amplified 16S rRNA genes, and sequences of the 16S-23S intergenic spacer regions (Seemuller et al., 2002). The taxonomic implications of these studies are that the phytoplasmas should be distinguished at the minimal taxonomic level of a genus, and each phytoplasma subclade should represent at least one distinct species (International Committee, 1997). However, in the absence of the phenotypic markers used to classify mollicutes, taxonomic affiliations cannot be resolved at the present time in the conventional way. Thus, a provisional classification of the uncultured phytoplasmas may be introduced by applying the Candidatus category proposed for classification of uncultured bacteria (International Committee, 1997). So far, seven Candidatus species names for phytoplasmas have been published (Seemuller et al., 2002; Table 1). The phytoplasma case demonstrates well the revolution occurring in bacterial taxonomy, where molecular data suffice for laying the phylogenetic and taxonomic basis for classification of a group of organisms for which very few phenotypic characteristics are available. On the other hand, the difficulty of basing classification on sequence data of uncultured organisms should not be ignored, as there is a danger that a mixture of phylogenetically diverse organisms may be considered erroneously as a single organism. Such chimeric sequences are easily recognized when reference sequences from cultured organisms are available for all components of the chimera. When such references are not available, it may be difficult to decide which of the new sequences represent those from a genuinely independent organism and which ones come from an artificial chimera.

The molecular approach to phytoplasma classification has recently been applied also to the uncultured hemoplasmas (Neimark et al., 2001; see the sections Habitats and Ecology) comprising the *Haemobartonella* and the *Eperythrozoon* species. These bacteria have been classified until recently as rickettsiae (order Rickettsiales) because of their small size, staining properties, obligate parasitism, and hemotrophic character. Several of them are transmitted by blood-feeding arthropods. However, phylogenetic analyses of 16S rRNA gene sequences have revealed that *Haemobartonella* and *Eperythrozoon* species are not rickettsiae but are rather mycoplasmas (Neimark and Kocan, 1997; Rikihisa et al., 1997) with the highest similarity scores with members of the pneumonia clade, closest to *M. fastidiosum* and *M. cavipharyngis* (Johansson et al., 1999; Neimark et al., 2002). Since the hemoplasmas have not been cultured as yet, naming of new species should be included in the Candidatus category (Neimark et al., 2001, 2002).

The morphology of the hemoplasmas is of small coccoid organisms attached to erythrocytes, while a few may appear free in the erythrocyte cytoplasm. The individual coccoid cells can aggregate to give the appearance of short chains and small clusters of rod-shaped organisms. Electron microscopy shows the coccoid organisms to be about 0.3 μm in diameter with no cell wall. This morphology corresponds well with that of mycoplasmas (Messick et al., 2002;

Neimark et al., 2002). Although some *Haemo-bartonella* and *Eperythrozoon* species can cause overt disease in healthy immunocompetent animals, the majority of these species produce chronic asymptomatic infections. Clinical disease usually includes hemolytic anemia that varies from mild to severe.

Although the 16S rRNA sequences have proved to be very effective tools in the phylogeny and taxonomy of mollicutes, the feeling is that additional phylogenetic markers are desirable to support the conclusions based on the 16S rRNA data. In fact, such markers have already been applied, including the conserved ribosomal protein genes (Gundersen et al., 1996), elongation factor EF-Tu (*tuf*) gene (Kamla et al., 1996), heat shock protein gene *hsp70* (Falah and Gupta, 1997), *ftsZ* gene (Kukekova et al., 1999), DNA repair *uvrC* gene (Subramaniam et al., 1998), ribonuclease P (*rnpB*) gene (Birkenheuer et al., 2002), *rpoB* gene encoding the β subunit of RNA polymerase (Kim et al., 2003), and 16S-23S rRNA intergenic sequences (Harasawa, 2000). Use of these markers has supplemented and complemented the 16S rRNA comparative data. A priori, wobble in the genetic code permits more variations in protein gene sequences, even of highly conserved genes, than is possible in rRNA sequences. Thus, even though the ribosomal protein-encoding genes are conserved, they vary in size and primary sequence more than the 16S rRNA genes. Therefore, the ribosomal protein genes have a greater potential to reveal variations among closely related strains. Likewise, the intergenic spacer region located between the 16S and 23S rRNA genes, being less subjected to evolutionary constraints than the genes themselves, exhibits greater sequence variation, a property of use in strain differentiation (Harasawa, 2000).

Generally, housekeeping or constitutive genes, which are expressed in all living organisms because they provide essential functions (such as replication, transcription and translation), are good candidates for genetic differentiation of species. The *uvrC* gene, which encodes deoxyribodipyrimidine photolyase, is well conserved within each species, but the gene is sufficiently different between species, so that it may serve as an effective taxonomic probe (Subramaniam et al., 1998). The heat shock Hsp70 family of proteins shows, perhaps, the highest degree of sequence conservation and is therefore well suited for examining deep phylogenetic relationships. The application of the *hsp70* gene to phylogenetic analysis of mollicutes has strengthened considerably the evidence gained through the 16S rRNA sequence analyses that mollicutes are evolutionarily closely related to the Gram-positive bacteria (Falah and Gupta, 1997).

Intraspecies Genetic Heterogeneity

Factors such as the arbitrary and somewhat vague definition of a bacterial species, the frequent and significant chromosomal rearrangements taking place in mycoplasmas (see the section Chromosomal Rearrangements), and the frequent integration of extrachromosomal elements into the mycoplasmal genome are conducive to the finding of intraspecies genotypic heterogeneity. Intraspecies genotypic heterogeneity, noticeable on serological testing and electrophoretic analysis of cell proteins, became much clearer through the application of more sensitive molecular tools such as restriction fragment length polymorphism (RFLP), restriction-modification activity profiles, and sequence analysis of conserved genes, species-specific protein genes, genomic physical maps, etc. (Razin, 1992; see the section Identification). These tests have provided epidemiologists with very sensitive tools to distinguish strains of interest in the clinic and in the field (see figures of RFLPs in the section Identification). On the basis of such molecular tools, the clinically important human *Ureaplasma urealyticum* species, long known to consist of two clusters of serovars, has recently been divided into two species, *U. urealyticum* and *U. parvum* (Robertson et al., 2001). Another example of clinical importance concerns the difference between the European and African-Australian strains of *M. mycoides* subsp. *mycoides* SC. The African-Australian strains are significantly more virulent than the European strains and cause more serious cases of contagious bovine pleuropneumonia. Recent studies indicate that the chromosome of the European strains lacks an 8.84-kb segment found in the African strains that includes two open reading frames (ORFs) of putative ATP-binding cassette (ABC) transporters and several genes encoding membrane proteins and an insertion element. This chromosomal deletion is associated with a deficiency in glycerol uptake and metabolism in the European strains, possibly explaining their lower pathogenicity (Vilei et al., 2000; see the section Pathogenicity).

Metabolic and Phenotypic Markers

The present tendency to depend on direct genomic analysis, made available by the dramatic advancements of molecular genetic methodology, has discouraged further development of classical taxonomic tools based on determination of nutritional requirements and enzymatic activities (Razin, 1992). Because they reflect the expression of specific genes and are thus liable to changes, electrophoretic cell

protein profiles are now used less extensively as taxonomic tools than they were in the 1970s and early 1980s. Protein genes themselves can now be sequenced and cloned and thus yield to more direct comparison. Yet, the recent development of the proteome approach to genomic analysis may revive the use of electrophoretic patterns of cell proteins as a taxonomic and phylogenetic tool (see the section Transcriptomes and Proteomes).

Note also that distinguishing members of the class Mollicutes by their metabolic characteristics has been generally of limited phylogenetic and taxonomic usefulness. A few exceptions include the ability to ferment glucose, to hydrolyze arginine and urea, and the dependence on cholesterol for growth and anaerobiosis. Cellular localization of reduced nicotinamide adenine dinucleotide (NADH) oxidase activity has also been of help in classification of mollicutes at and above the genus level. Though *Acholeplasma* species resemble other prokaryotes in having the NADH oxidase localized in the plasma membrane, this enzymatic activity is located in the cytoplasm of *Mycoplasma*, *Spiroplasma* and *Ureaplasma* species. The finding of this activity in the cytoplasm of the *Mesoplasma* and *Entomoplasma* species has helped to distinguish these newly described genera from *Acholeplasma* species (Tully et al., 1993; Pollack et al., 1996). Extensive studies carried out in the laboratory of J. D. Pollack (reviewed in Pollack [2002b]) were aimed at the finding of mycoplasmal enzymatic activities useful for the differentiation of mollicutes genera. Several activities (endowed with such a potential) were defined but so far not put into general use.

At the species level, serologic relatedness has until recently overshadowed all other features used in routine mollicute identification, but the weight given to determination of molecular properties in mollicute classification and identification is steadily increasing (Razin, 1992). The high rate of surface antigenic variation characterizing mollicutes (see the section Antigenic Variation) may impose some limitations on the use of monoclonal antibodies to surface antigens as tools in mycoplasma identification (Rosengarten and Yogev, 1996), difficulties usually not encountered when polyclonal antibodies are used (International Committee, 1997, 2000). To limit the number of serological tests and more so the large number of specific antisera currently required for the definition of new mollicute species, the International Committee on the Taxonomy of Mollicutes (2001) has agreed that the new isolate can be placed in the proper phylogenetic clade on the basis of 16S rRNA sequences and serological cross-testing can be restricted to members of this clade.

The weight to be given to cholesterol requirement in mollicute taxonomy illustrates well the conflict that may occur between taxonomy based on phenotypic characteristics and that based on molecular phylogenetic data. Cholesterol requirement has been considered for a long time as a major criterion in establishing high taxonomic groupings within the Mollicutes (Razin and Freundt, 1984a). Recent findings weaken the high status of cholesterol requirement in mollicute classification. Thus, the cholesterol-nonrequiring mesoplasmas, considered previously as acholeplasmas, were shown to possess molecular properties very different from those of the classic acholeplasmas, requiring their taxonomic separation (Tully et al., 1993; Pollack et al., 1996). In addition, several *Spiroplasma* species were shown to grow in the absence of cholesterol (Tully et al., 1993). These data suggest that nutritional dependency on exogenous sterols is a trait that has arisen independently several times during the evolution of mollicutes, as it occurs in at least three different phylogenetic groups. Thus, cholesterol requirement apparently should not be used as a sole definitive criterion in defining higher taxa of the class Mollicutes but rather serve as one of a matrix of characters in mollicute classification. Consequently, though nutritional requirements are the most practical traits used to assess in routine laboratory tests, they may not always be reliable indicators of phylogenetic relationships.

Blending Taxonomy with Phylogeny

Whereas basing bacterial taxonomy on phylogeny is generally thought to be both inevitable and advantageous, bacterial taxonomists disagree about whether the time is ripe for radical changes in bacterial taxonomy (for detailed discussion, see Razin [1992]). The taxonomic status of the *Mycoplasma mycoides* cluster illustrates well the problem. The Mollicutes phylogenetic tree based on 16S rDNA sequences has suggested that the *M. mycoides* cluster is phylogenetically related to the genus *Spiroplasma* (Weisburg et al., 1989). However, the proposal to reclassify this cluster accordingly has not been adopted by the International Committee (1997), arguing that such a reclassification would create considerable problems in diagnostic veterinary medicine. Likewise, reassignment of all other *Mycoplasma* species to a new genus would have unacceptable consequences in human and veterinary medicine. For the time being, a polyphasic classification based only partly on phylogeny is recommended, striving to fit in mollicute taxa as much as possible into phylogenetic classification.

Habitats and Ecology

Mycoplasmas are widespread in nature as parasites of humans (Blanchard and Bebear, 2002), mammals (Frey, 2002), reptiles (Brown et al., 2001), fish, arthropods and plants (Tully, 1996; Seemuller et al., 2002). The list of hosts known to harbor mycoplasmas is continuously increasing, as does the number of established mollicute species (about 200 so far; Table 1). It is widely agreed that the mollicutes that have already been characterized and taxonomically defined constitute only a part, apparently a minor one, of the mollicutes living in nature (Razin, 1992). The higher the number of established mollicute species, the harder it gets to fulfill the minimal requirements for establishing new species (International Committee, 1995). A large battery of species-specific antisera and seed is needed for this task. The introduction of molecular tools to taxonomy, including the comparison of 16S rRNA and of other conserved gene sequences, genomic restriction patterns, etc. (Razin, 1992, 1994) has already been found effective in species and strain identification, decreasing the weight of serology in species and strain identification and classification (see the sections Phylogeny and Taxonomy and Identification).

Uncultured Mollicutes

The successful application of genomic analysis methodology has led to the identification of the uncultured plant and insect MLOs as bona fide mycoplasmas (see Phylogeny and Taxonomy), showing how the identity of other mycoplasma-like organisms can be confirmed as mollicutes. Thus, the grey lung virus infection of mice, caused by a mycoplasma-like agent (as seen in thin sections of lung material), but resisting cultivation, has been identified as a mycoplasma on the basis of its genome size and 16S rRNA sequence. Classification of this organism as "Candidatus *Mycoplasma ravipulmonis*," the "Candidatus" taxon being reserved for classification of uncultured prokaryotes (Neimark et al., 1998), has been proposed. Most important are the findings, based on 16S rRNA sequence analysis, that *Haemobartonella* and *Eperythrozoon* (well-known uncultured wall-less pathogenic bacteria, infecting erythrocytes of a wide range of vertebrate hosts) are phylogenetically closely related to Mollicutes rather than to rickettsia and should therefore be reclassified as *Mycoplasma* species (Neimark and Kocan, 1997; Rikihisa et al., 1997). According to a 16S rRNA phylogenetic tree (Fig. 12), these two hemotropic bacteria are closest to the *M. fastidiosum–M.*
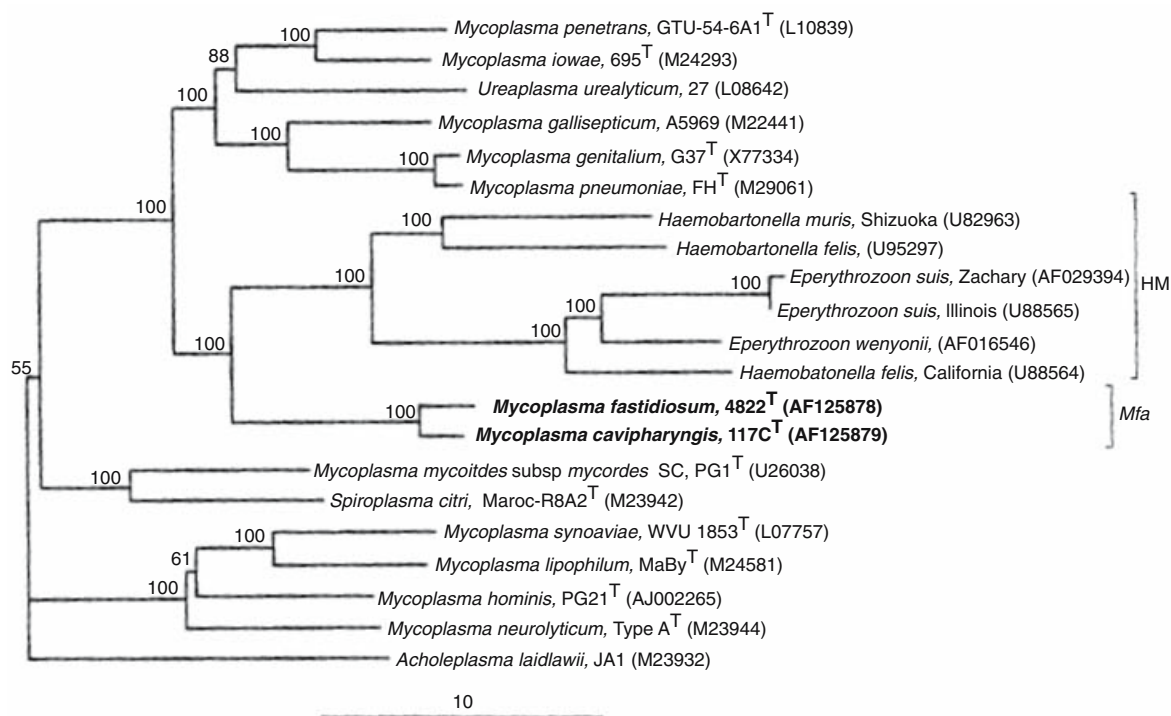
Fig. 12. Phylogenetic tree based on 16S rRNA sequences showing the relations between *Eperythrozoon* spp. and *Haemobartonella* spp. and mycoplasmas. According to the dendogram, the hemotropic bacteria are closest to the *M. fastidiosum–M. cavipharyngis* cluster. From Johansson et al. (1999).

cavipharyngis cluster (Johansson et al., 1999). The inclusion of these hemotropic agents in Mollicutes represents an entirely new group of pathogens among the mycoplasmas (see also the section Phylogeny and Taxonomy).

Host and Tissue Specificity

Mycoplasmas usually exhibit a rather strict host and tissue specificity, probably reflecting their nutritionally exacting nature and obligate parasitic mode of life. However, there are numerous examples of mycoplasmas present in hosts and tissues different from their normal habitats (Tully, 1996). The primary habitats of human and animal mycoplasmas are the mucous surfaces of the respiratory and urogenital tracts, the eyes, alimentary canal, mammary glands, and the joints. The obligatory anaerobic anaeroplasmas have so far been found only in the bovine and ovine rumen (Table 1). Spiroplasmas and phytoplasmas are widespread in the gut, hemocoel, and salivary glands of arthropods. The spiroplasmas and phytoplasmas may be introduced through sap-sucking insects to the phloem tissues of plants, causing disease (Tully and Whitcomb, 1992; Seemuller et al., 2002). Accompanying the increase in the number of patients suffering from various types of immunodeficiencies, associated with hypogammaglobulinemia, acquired immune immunodeficiency syndrome (AIDS), and treatment with immunosuppressive medication is the increase in the number of reports on the isolation of mycoplasmas from organs different from their usual habitats. Thus, mycoplasmas and ureaplasmas belonging to the normal urogenital flora have been isolated from the blood of patients suffering from AIDS or treated with immunosuppressive drugs. Hypogammaglobulinemic and immunocompromised patients become susceptible to infections by the urogenital mycoplasmas *M. hominis* and *U. urealyticum*, spreading into and causing disease in organs such as the respiratory tract and joints (Gass et al., 1996; Frangogiannis and Cate, 1998).

Mycoplasmas Infecting Cell Cultures

Cell cultures infected by mycoplasmas constitute an artificial unnatural habitat. The serious problems created by the persistent and hard to detect infections of cell cultures are reflected in the voluminous literature on this subject. Reports from various countries show that 10–87% of cell cultures are infected by mycoplasmas (Barile and Rottem, 1993; Uphoff and Drexler, 2002). The percentage of infected cell cultures depends to a large extent on the population of cell cultures assayed, control practices used, and

efficiency of the assay procedures applied. The mycoplasma species infecting cell cultures have remained essentially the same, with *M. hyorhinis*, *M. orale*, *M. arginini* and *A. laidlawii* being the dominant contaminants. *Mycoplasma fermentans*, a mycoplasma suspected to be a cofactor in AIDS (see the section Disease Manifestations), is increasingly found as a contaminant infecting cell cultures and is therefore of particular interest in studying the possible origin of contaminating mycoplasmas. Elimination of mycoplasmas from infected cell cultures by antimycoplasmal agents is time-consuming and often unsuccessful (Uphoff et al., 2002) probably because a small percentage of the contaminating mycoplasmas survive in an intracellular location.

The hard-to-detect mycoplasma contamination of stock virus and chlamydia kept in cell cultures could, of course, pose serious problems when these stock cultures are used as seed and reference antigens (Huniche et al., 1998). Mycoplasmas infecting the cell cultures were shown to produce or induce numerous factors and enzymatic activities reported in the cultures. For example, a protein detected in tumor cell lines, named "M161Ag" activated human complement and induced proinflammatory cytokines. This protein, originally thought to be produced by the cell line, was later shown to originate in *M. fermentans* latently infecting the tumor cell cultures (Matsumoto and Seya, 1999). Tests based on conversion of radiolabeled arginine to citrulline for the presence of inducible nitric oxide synthase in neonatal foreskin fibroblast cultures, were compromised by the presence of contaminating mycoplasmas that convert arginine to citrulline by the arginine dihydrolase system (Choi et al., 1998). Thus, the need to take all precautions to prevent mycoplasma contamination of cell cultures cannot be overemphasized.

Human and Animal Mycoplasmas

Table 2 lists the mycoplasmas found in humans. Some of these mycoplasmas may cause disease whereas others are commensals, constituting part of the normal flora of the oropharynx and the urogenital tract. Some of the human mycoplasmas shown in Table 2 can also be isolated from nonhuman primates.

Farm animals, cattle, sheep, goats, horses, swine, chickens and turkeys harbor a great variety of mollicutes, many of them pathogenic. Mollicutes can be found also in dogs, cats, small laboratory animals, either as commensals or pathogens. A mollicute flora has been defined in a wide variety of wild animals, including elephants, lions, seals, crocodiles, turtles and

Table 2. Common human mollicutes.[a]

Species	Primary organ	Pathogenicity
M. pneumoniae	Lung, oropharynx	Primary atypical pneumonia
M. genitalium	Urogenital tract	Nongonococcal urethritis in men and PID in women
M. hominis	Urogenital tract	Usually commensal, may cause infections on reaching internal organs (salpingitis)
M. fermentans	Urogenital tract, respiratory tract	Cofactor in AIDS activation? Arthritis?
M. penetrans	Urogenital tract? Colon?	Cofactor in AIDS activation?
M. salivarium	Buccal cavity	Commensal. Periodontal disease?
M. orale	Buccal cavity	Commensal
Ureaplasma urealyticum and Ureaplasma parvum	Urogenital tract, placenta	Nongonococcal urethritis, neonatal lung and brain infections

Abbreviations: PID, pelvic inflammatory disease; and AIDS, acquired immunodeficiency syndrome.
[a]Commensal mycoplasmas less commonly isolated from humans: *M. buccale*, *M. faucium* and *M. lipophilum* from the oropharynx; *M. primatum* and *M. spermatophilum* from the genitals and *M. pirum* from blood (see Blanchard and Bebear [2002]). A novel, yet unnamed, *Mycoplasma* species has recently been isolated from sputa of immunocompromised patients suffering from chronic bronchitis (Webster et al., 2003).

prawns. In essence, mollicutes may be ubiquitous in the animal world. Apparently the main factor determining whether an animal is added to the long list of animals harboring mycoplasmas is the willingness of the mycoplasmologist to isolate and characterize the mycoplasmas from the animal. Interestingly, the laboratory rabbit represents an exception among the animals so far studied, as it appears to be free of mycoplasmas. Lack of a natural mycoplasma flora makes the rabbit a preferred animal for production of specific antisera to mycoplasmas. For detailed descriptions of the mollicute flora of humans and animals, see Blanchard and Bebear (2002) and Frey (2002).

Mycoplasmas in Arthropods and Plants

Arthropods are major habitats of mollicutes, including the helical spiroplasmas, sterol-nonrequiring mesoplasmas, sterol-requiring entomoplasmas, and the uncultured phytoplasmas (Table 1). The spiroplasma group is apparently one of the most abundant groups of microbes. Spiroplasmas have been reported from species of six evolutionary advanced insect orders: *Hymenoptera* (honeybees and wasps), *Coleoptera* (beetles), *Diptera* (flies and blood-sucking insects), *Lepidoptera* (butterflies), *Homoptera* (leafhoppers), and *Hemiptera* (leaf bugs). Spiroplasmas were also isolated from ticks, but it is not clear whether spiroplasma occurrence relates to the developmental stages of the tick or whether vertebrates play a role in maintaining the spiroplasmas in the ticks (Tully and Whitcomb, 1992; Gasparich, 2002).

Classification of spiroplasmas was based for a long time mostly on grouping, and official binomial names were coined with caution. This policy has been changed, already yielding 34 named *Spiroplasma* species (Table 3), and this number is expected to increase to at least 40 (International Committee, 2000).

Spiroplasmas are most frequently found in the insect gut, less frequently in the hemolymph, and occasionally in various organs such as the salivary glands. Spiroplasmas in the insect gut are generally acquired by natural feeding, either from plant tissue, nectar, or ingestion of other insects. Some spiroplasmas, like the honeybee spiroplasmas, are pathogenic and may kill their insect hosts or eliminate the male progeny of infected *Drosophila* (Williamson et al., 1999), whereas others are considered commensals, even when present in large numbers in the hemolymph (Table 3). Spiroplasmas involved in vector-transmitted plant diseases (citrus stubborn and corn stunt) must pass through a complex biological cycle that involves uptake from the sieve cells of the plant phloem and subsequent passage to, or multiplication in, the insect alimentary tract, gut epithelium, basement membrane, hemocoel, and possibly some internal organs. At this phase of infection the spiroplasmas lose their helicity and appear as rounded, pleomorphic bodies with a reticulated internal structure, most frequently found in membrane-bound pockets in the insect cell (Kwon et al., 1999). The organisms eventually pass from the hemocoel into the ciliary cells and salivary duct, from which reinoculation of healthy plants takes place. Induction of disease in the plant is the rule. The symptoms in plants include chlorosis, leaf mottling, proliferation of growing points and general stunting (Tully and Whitcomb, 1992).

Many spiroplasmas observed in the hemolymph of insects have resisted cultivation. The sex-ratio spiroplasma of *Drosophila* spp., one of the first spiroplasmas to be described, was cultivated in vitro following the initial cocultivation with insect cell lines (see Isolation).

Table 3. *Spiroplasma* species, their hosts and disease manifestations.

Species	Principal host	Disease manifestations
S. alleghenense	*Panorpa helena* scorpion fly	U
S. apis	Bees and flowers	"May disease" of bees
S. cantharicola	*Cantharis* beetle	U
S. chinense	*Calystegia hederaceae* flowers	U
S. chrysopicola	*Chrysops* sp. deerfly	U
S. citri	Dicots and *Circulifer* leafhoppers	Citrus stubborn
S. clarkii	*Cotinus* beetle	U
S. corrscae	*Ellichnia corrusca* firefly	U
S. culicicola	*Aedes* mosquito	U
S. diabroticae	*Diabrotica undecimpunctata* corn rootworm beetle	U
S. diminutum	*Culex annulus* mosquito	U
S. floricola	Insects and flowers	U
S. gladiatoris	*Tabanus gladiator* horsefly	U
S. helicoides	*Tabanus abactor* horsefly	U
S. insolitum	*Eristalis* fly and flowers	U
S. ixodetis	*Ixodes pacificus* ticks	U
S. kunkelii	Maize and leafhoppers	Corn stunt
S. lampyridicola	*Photuris pennsylvanicus* firefly beetle	U
S. leptinotarsae	*Leptinotarsa decemlineata* (Colorado potato beetle)	U
S. lineolae	*Tabanus lineola* horsefly	U
S. litorale	*Tabanus nigrovittatus*	U
S. melliferum	Bees	Honey bee spiroplasmosis
S. mirum	*Haemaphysalis* rabbit ticks	Suckling mouse cataract disease[a]
S. monobiae	*Monobia* wasp	U
S. montanense	*Hybomitra opaca* horsefly	U
S. phoeniceum	*Catharanthus roseus*	Periwinkle disease
S. platyhelix	*Pachydiplax longipennis* dragonfly	U
S. poulsonii	*Drosophila willstoni*	Male lethality[b]
S. sabaudiense	*Aedes* mosquito	U
S. syrphidicola	*Eristalis arbustorum* syrphid fly	U
S. tabanidicola	*Tabanus abdominalis limbatinervis* horsefly	U
S. taiwanense	*Culex tritaeniorhynchus* mosquito	U
S. turonicum	*Haematopta* horsefly	U
S. velocicresens	*Monobia* wasp	U

Abbreviation: U, not known.
[a]Experimental.
[b]Sex-ratio trait.
Data from Williamson et al. (1998).

Spiroplasma occurrence in plant hosts is based on either invasion of the plant sieve tubes in the course of a biological cycle involving the feeding of homopterous insects (leafhoppers) or on external contamination of floral parts deposited by flower-visiting insects. Only spiroplasma invasion into plant sieve tubes results in disease, as seen in various citrus plant diseases with *S. citri*, in corn stunt disease with *S. kunkelii*, and in the so-called "periwinkle disease" with *S. phoeniceum*. Numerous other *Spiroplasma* species have been isolated from flower surfaces and nectar, but no evidence is currently available that these organisms also occur within internal plant tissues (Table 3). However, some normal flower-inhabiting spiroplasmas acquired by specific insects act as pathogens, as is the case of *S. apis* strains acquired by honeybees (Tully, 1996). A rather intriguing issue concerns the possible occurrence and pathogenicity of spiroplasmas in animals. Only one spiroplasma, *S. mirum* strain SMCA has been shown to experimentally infect suckling mice causing cataracts (Gasparich 2002). Claims associating spiroplasmas with transmissible human spongiform encephalopathies have been repeatedly made, the most recent claim being based on positive PCR tests for *S. mirum* 16S rDNA in human brain samples (Bastian and Foster, 2001). Spiroplasma-like structures could also be demonstrated in a cataract in a premature baby, a finding supported by positive PCR with the 16S rDNA of *Spiroplasma* group VI (Lorenz et al., 2002). The above findings may be considered at most suggestive but appear worthy of further study.

The nonhelical mollicutes of the genera *Entomoplasma* and *Mesoplasma* have been found to inhabit the gut of insects (fireflies,

beetles, horseflies, moths, butterflies and bees). However, some entomoplasmas and mesoplasmas have been identified also on plant surfaces, so that most species in either group can be acquired from various plant surfaces or flowers through insect visitation and feeding excursions (Tully, 1996). The current distribution of *Mesoplasma* species, which were initially described as acholeplasmas (Tully et al., 1993), has been summarized in Tully et al. (1994). Again, most isolates have been made from gut fluids of insect hosts, with a few isolations from plant surfaces.

The largest group of mollicutes inhabiting plants is the currently uncultured plant-pathogenic phytoplasmas. These mollicutes are transmitted primarily by phloem-feeding leaf-hoppers and are the causative agents of more than 300 different plant yellows diseases. For a detailed description of the phytoplasma groups, the diseases they cause, their plant hosts, insect vectors, and geographic distribution, see the reviews of Seemuller et al. (2002) and Lee et al. (2000). The different geographic location of any specific phytoplasma depends on the availability of not only the plant host but also the insect vector in the area. Plants infected by phytoplasmas exhibit an array of symptoms that suggest profound disturbances in the normal balance of plant hormones or growth regulators. Symptoms include virescence (green flowers), phyllody (floral parts develop into leafy structures), sterility of flowers, proliferation of auxiliary shoots (witches broom appearance), abnormal elongation of internodes resulting in slender shoots, generalized stunting, discoloration of leaves, and yellowing (Lee et al., 2000; Seemuller et al., 2002).

The phytoplasma vectors belong to the families Cicadelloidae (leafhoppers) and Fulgoroidae (plant hoppers). Some phytoplasmas have low insect vector specificity, whereas others have very high vector specificity. Plant host range for each phytoplasma is largely determined by the number of natural insect vector species capable of transmitting the phytoplasma and by their feeding behavior. Many vectors can transmit more than one type of phytoplasma, and many plants can harbor two or more distinct phytoplasmas. Periwinkle, commonly used as a source plant to maintain phytoplasma cultures, is able to harbor the majority of known phytoplasmas. Overlapping vectors and plant hosts have allowed ample opportunities for phytoplasmas to interact with one another and exchange genetic information. Exchange may occur vertically and horizontally. As a result, a widely diverse phytoplasma group has been formed with characteristics of continual and wide genetic variation among its members (Lee et al., 2000).

Isolation

Several host and cultural factors are important in primary isolation and maintenance of mycoplasmas during early passage levels. These include the chemical and physical suitability of the culture medium, the number of organisms in the tested specimen, and the presence of toxic or inhibitory substances in the inoculum (Tully and Whitcomb, 1992). The use of antibiotics and thallium acetate in culture media has made possible mycoplasma isolation in the presence of a variety of other competing organisms in the tested material. Penicillin (500–1000 units/ml) and polymyxin B (500–1000 units/ml) have been the drugs of choice for inhibition of either Gram-positive or Gram-negative prokaryotes and fungi, respectively (Tully, 1983). Thallium acetate toxicity to man and animals and its possible growth inhibition of some mollicutes, such as ureaplasmas, at the concentration recommended in mycoplasma culture media (about 1 mg/ml) has led to the suggestion (Angulo et al., 2003) that colistin sulfate (37 μg/ml) be used instead. Clearly, the effectiveness of colistin sulfate as a selective agent for mycoplasmas should be tested in clinical diagnostic laboratories prior to replacement of thallium acetate. Mycoplasmas can often be isolated from diluted but not undiluted infected tissue extracts, suggesting that inhibitors like lysolecithin, antimycoplasmal antibodies, and antibiotics are present in host tissues (Taylor-Robinson and Chen, 1983). It is advisable, therefore, to perform serial dilutions of such extracts in fresh culture media in all primary isolation attempts. Early broth passages of newly isolated mycoplasmas are likely to require prolonged incubations rather than the shorter time required for strains well adapted to a particular medium.

The first indication of mycoplasma growth in primary broth cultures is often a slight-to-moderate pH change of the medium, and in most instances, slight turbidity. Phase-contrast and dark-field microscopy may be effective in monitoring broth cultures, particularly in the case of the motile helical spiroplasmas (Tully and Whitcomb, 1992). Checking the agar cultures for the typical fried-egg colonies under a stereomicroscope is the next step. Frequently, the shape of the colonies is not typical. Thus the motile spiroplasmas may form diffuse colonies on the fresh agar surface, lacking the agar-embedded central zone. By increasing the agar concentration, the free water film on the agar surface decreases in thickness (Razin and Oliver, 1961). This impedes spiroplasma motility in the film, resulting in the formation of fried-egg colonies (Tully and Whitcomb, 1992).

Culture Media

A major impediment to mycoplasma research and laboratory diagnosis of mycoplasma infections has been the difficulty of their in vitro cultivation. The consensus is that only a minority of the mycoplasmas existing in nature has been cultivated so far. For example, despite many efforts for over 30 years, none of the phytoplasmas infecting insects and plants has been cultivated in vitro in an axenic culture (Seemuller et al., 2002). Some cultivable mycoplasmas (such as the human respiratory pathogen *M. pneumoniae*) grow very slowly (2–3 weeks incubation at 37°C), particularly on primary isolation. Others, like *Ureaplasma urealyticum*, grow very fast in vitro, but growth stops abruptly at titers of 10^6–10^7 colony-forming units per milliliter (cfu/ml) compared to 10^9–10^{10} cfu/ml in logarithmic-phase cultures of well-growing mycoplasmas (Fig. 13). The recent mycoplasma genome sequencing projects have provided definitive genetic explanations to the above-mentioned difficulties by demonstrating the remarkable scarcity in mycoplasmas of genes involved in biosynthetic pathways. For example, both *M. genitalium* and *M. pneumoniae* lack all the genes involved in amino acid synthesis (Fraser et al., 1995; Himmelreich et al., 1996), making them totally dependent on the exogenous supply of the complete spectrum of amino acids (see Genome Sequencing and Gene Annotation).

To overcome the assimilative deficiencies of the mycoplasmas, complex media are used for their cultivation. The media are usually based on beef heart infusion, peptone, yeast extract and serum with various supplements (see media formulations below). Use of these complex undefined growth media has interfered with the molecular definition of mycoplasmal metabolic pathways, genetic analysis, preparation of mycoplasmal antigens free of serum components, etc. The aim of efforts to replace the serum component was a defined growth medium. Serum has been shown to provide, among other nutrients, fatty acids and cholesterol required for membrane synthesis, in an assimilable, nontoxic form. Serum component substitution by albumin, fatty acids, cholesterol solubilized in Tween 80, or liposomes made of phospholipids and cholesterol, and the addition of serum albumin to neutralize free fatty acid toxicity has met with only limited success (Razin, 1978). Defined culture media were developed for only a few fermentative mycoplasmas. The media contain a complex assortment of amino acids, nucleic acid precursors, lipids, vitamins, inorganic ions, together with glucose as an energy source (Rodwell, 1983; Hackett et al., 1987). Detailed composition of media for mycoplasmas can be found in the section on cultivation and nutrition in *Methods in Mycoplasmology* (Razin and Tully, 1983), in Tully and Whitcomb (1992), Tully (1995a), and in Hackett and Whitcomb (1995).

Composition of some of the more common media for cultivation of mycoplasmas is given below:
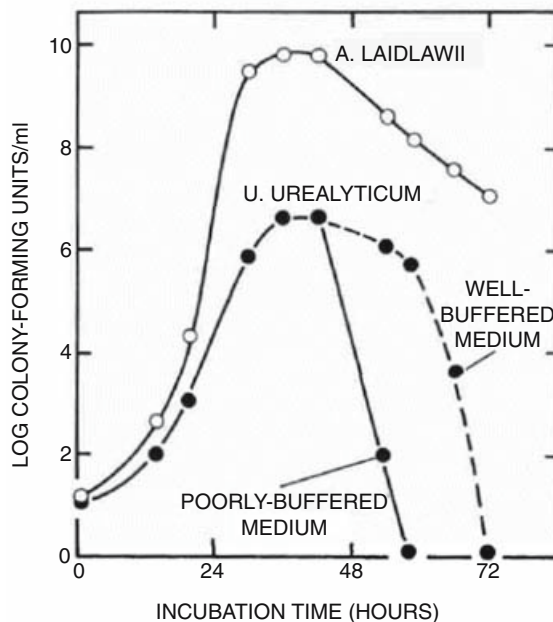
Fig. 13. Growth curves of *Ureaplasma urealyticum* compared to that of *Acholeplasma laidlawii*. From Razin (1978).

Modified Hayflick Medium (Freundt, 1983)

Heart infusion broth (Difco)	28.5 g
Deionized water	900 ml
Fresh yeast extract (25%, w/v)	100 ml
Horse serum	200 ml
Calf thymus DNA (0.2%, w/v)	12 ml
Thallium acetate (1%, w/v)	10 ml
Penicillin G (20,000 units/ml)	2.5 ml

Supports good growth of the majority of *Mycoplasma* and *Acholeplasma* species.

Dissolve the heart infusion broth in deionized water and autoclave (121°C for 20 min). Aseptically add the remaining sterile solutions. Adjust final pH to 7.8 with sterile NaOH solution.

Medium SP-4 (Tully, 1995a)

Mycoplasma broth base (Becton-Dickinson, Baltimore, MD)	1.4 g
Tryptone (Difco)	4.0 g
Peptone (Difco)	2.13 g
Deionized water	270 ml
Phenol red solution (0.1% aqueous solution)	8 ml

Use this medium to grow fastidious mycoplasmas and spiroplasmas. Heat to dissolve ingredients and adjust pH to 7.8 (with about 3–4ml of 1N NaOH). Sterilize at 121°C for 20min. For solid medium, add 0.8g of purified agar (Noble, Difco) for each 100-ml volume of broth and omit the phenol red indicator. Add ingredients (below) as sterile supplements. Adjust final pH of the complete medium to 7.6–7.8 and to a final volume of 400ml.

CMRL 1066 tissue culture medium with glutamine (10X, Gibco)	20ml
Glucose (50% aqueous solution)	4ml
Fresh yeast extract (25% aqueous solution, Gibco)	14ml
Yeastolate (4% aqueous solution, Difco)	20ml
Fetal bovine serum (Hyclone Laboratories)	68ml
Penicillin G (100,000 units/ml)	2ml

Heat-inactivate the fetal bovine serum at 56°C for 1h.

For detailed description of the preparation procedure of the SP-4 medium, see Tully (1995a). Additional undefined and defined media for spiroplasma cultivation are described by Tully and Whitcomb (1992) and by Hackett and Whitcomb (1995b).

U9C Urea Medium (Shepard and Lunceford, 1976)

Trypticase soy broth (BBL)	15.0g
Magnesium chloride (MgCl$_2$·6H$_2$O)	0.2g
Yeast extract (Difco)	1.0g
Deionized water	900ml

Supports growth of ureaplasmas and indicates urea hydrolysis. Adjust pH to 5.5 and autoclave at 121°C for 15min. Add aseptically the following sterile solutions.

Urea (10%, w/v)	3ml
L-Cysteine-HCl (2%, w/v)	5ml
GHL tripeptide (Gly-His-Lys, Calbiochem, 20g/ml)	1ml
Horse serum	100ml
Penicillin G (100,000 units/ml)	10ml
Phenol red (1% w/v)	1ml

Adjust the final pH to about 6.0.

The definition of lipid requirements, particularly for cholesterol, has served as an important taxonomic criterion distinguishing the sterol-nonrequiring mollicutes, particularly the *Acholeplasma* species, from the sterol-requiring taxonomic entities. Testing for sterol requirement has been based on the use of a serum-free medium containing serum albumin, fatty acids and various concentrations of cholesterol solubilized in Tween 80, yielding a final Tween concentration of 0.01% (Tully, 1995b). The finding (Rose et al., 1993) that Tween 80 at a final concentration of 0.04% is essential for growth of some of the sterol-nonrequiring mycoplasmas (later named *Mesoplasma* species; Table 1) is still an enigma. It seems unlikely that requirement for this relatively high Tween 80 concentration can be explained by provision of a required fatty acid component, inasmuch as experiments to test this issue by adding a variety of fatty acids have failed.

Although the numerous nutritional requirements of mollicutes dictate the need for complex growth media, the notion that the richer the medium the better, may be wrong. Apparently, at least in some cases, the lack of growth of a mycoplasma in a rich medium is not due to the lack of a specific nutrient but rather to the presence of a component(s) toxic to the mycoplasma. Some *M. hyorhinis* strains, common contaminants of cell cultures, have been known to resist cultivation on conventional mycoplasma media, suggesting that these strains were particularly fastidious. This concept proved to be wrong because these strains could grow well in a minimal serum-free medium. Gardella and Del Guidice (1995) and Del Giudice (1998), therefore, proposed that these "noncultivable" strains are not particularly fastidious but more sensitive to inhibitors found in the complex media, mostly as components of peptone and yeast extract. Hence, "noncultivable" and "fastidious" should be considered as relative terms that take their meaning only in the context of a specific culture system where growth promoters, and possibly inhibitors, are present.

Generally, mollicutes differ markedly in their atmospheric requirements; whereas most mollicutes are facultative anaerobes, and usually favor an anaerobic or low-redox-enhanced CO$_2$ atmosphere on primary isolation, the rumen *Anaeroplasma* and *Asteroleplasma* species are strict anaerobes, very sensitive to oxygen (Table 1). Some mycoplasmas, such as *M. hyorhinis* require an aerobic atmosphere (Gardella and Del Guidice, 1995). The initial pH of the growth medium should be adjusted to about 8.0 for the fermentative mycoplasmas, and to 6.0–6.5 for the nonfermentative arginine-utilizing mycoplasmas and urea-hydrolyzing ureaplasmas. The temperature for mycoplasma growth ranges according to species from about 20–40°C. The optimum temperature for most human and warm-blooded animal mycoplasmas is 36–37°C, while the optimum growth temperature for *M. mobile*, isolated from fish, is 25°C (Kirchhoff et al., 1987) and 30°C for the tortoise mycoplasmas, *M. agassizi* and *M. testudinis* (Brown et al., 2001). Likewise, the optimum temperature for growth of the sterol-requiring entomoplasmas and the sterol-nonrequiring mesoplasmas (isolated from insects and plants) is about 30°C, while the optimum growth temperature of the spiroplasmas and the acholeplasmas is 30–37°C (Tully et al., 1993).

Cocultivation with Cell Cultures

A novel approach to improve the chances of in vitro cultivation of fastidious mollicutes is based

on coculture with eukaryotic cell lines (cell-assisted growth). In this way uncultivable spiroplasmas, such as the Colorado potato beetle spiroplasma (*S. leptinotarsa*) and the *Drosophila* sex-ratio spiroplasma (*S. poulsonii*) were successfully cocultivated first in insect cell lines, and then primary cultures of this spiroplasma were obtained on cell-free media under conditions of low redox, enhanced CO_2 atmosphere, and at a pH lower than 7.0 (for most mollicutes, the initial pH is adjusted to a slightly alkaline value)—the same conditions used in the insect cell cocultures (Hacket and Lynn, 1995; Konai et al., 1996). Though cocultivation of fastidious spiroplasmas with arthropod cell lines has proved itself in these cases, the choice of an adequate medium for primary isolation and subsequent sustained cultivation of these organisms is still much more an art than a science. However, all of the extensive knowledge and techniques that succeeded in primary isolation and cultivation of spiroplasmas have failed so far when applied to cultivation of the plant and insect phytoplasmas.

Cell-assisted cocultivation, using Vero cell cultures, enabled the cultivation of several strains of the highly fastidious human *M. genitalium* from clinical specimens (Jensen et al., 1996). Also in this case, the mycoplasmas grown in cell culture (as was indicated by PCR monitoring) could be subsequently subcultured in a cell-free medium. The above methodology, as complex as it is, requiring heroic efforts, is certainly inadequate for routine cultivation of fastidious mycoplasmas, leaving the door open for the application of molecular techniques, such as PCR, for detection and identification of fastidious, or the so far uncultivable mycoplasmas (see Identification and Taxonomy and Phylogeny).

Identification

Mycoplasma identification and laboratory diagnosis of mycoplasmal infections has been based on the classical bacteriological tests (i.e., morphology, cultural characteristics, physiological and serological properties). Though these tests still play a major role in mycoplasma diagnostics, new tests based on the molecular analysis of genomic DNA, ribosomal RNAs, cell proteins, and lipids appear to have replaced the classical tests (Razin, 2002). In addition, these molecular tests may soon become the prevailing methods for mycoplasma identification. Systematic and detailed description and evaluation of the classical procedures used in mycoplasma identification can be found in the two volumes of *Methods in Mycoplasmology* (Razin and Tully, 1983; Tully and Razin, 1983), whereas the newer molecular methods are included in the two more recent

volumes of *Molecular and Diagnostic Procedures in Mycoplasmology* (Razin and Tully, 1995; Tully and Razin, 1996). See also the concise review of current methods applied to the routine laboratory diagnosis of mycoplasmal infections (Waites et al., 2001).

Cultural Properties and Biochemical Tests

The International Committee on the Taxonomy of Mollicutes (a subcommittee of the International Committee on Systematic Bacteriology) issued recommended tests for mycoplasma identification as well as for description of new species of the class Mollicutes (International Committee, 1995). The recommended tests include those required to define a new isolate at the higher taxonomic levels (class, order and family) as well as tests for genus and species determination. Thus, a test for sterol requirement that depends on growth promotion by cholesterol in the presence of either 0.01% or 0.04% Tween 80 (Tully, 1995b) separates the sterol-nonrequiring *Acholeplasma* and *Mesoplasma* species from the members of Mollicutes that require cholesterol for growth. *Ureaplasma* identification is based on urea hydrolysis tests (Razin, 1983). Other key tests include those for sugar fermentation and arginine hydrolysis (Razin and Tully, 1983).

Serology

A great variety of serological tests have been employed, particularly for mycoplasma species and strain identification. The classical, recommended tests include growth and metabolism inhibition by specific antisera as well as direct and indirect immunofluorescence tests applied to mycoplasma colonies. A combined deformation-metabolism inhibition test system has been used to provide both screening and refined analysis and definition of *Spiroplasma* species (see the relevant chapters in *Methods of Mycoplasmology*; Tully and Razin [1983]). A variety of other, in some cases more sensitive, tests based on principles of enzyme-linked-immunosorbent assay (ELISA), immunobinding, immunoblotting, and immunoperoxidase tests, employing polyclonal or monoclonal antibodies, have been applied to mycoplasma diagnosis (see section B chapters in Tully and Razin [1996]). Owing to their higher sensitivity and specificity, these tests are capable of identifying strains within a species. One particular advantage of the colony immunofluorescence, immunoperoxidase and immunobinding techniques is their ability to distinguish a certain mycoplasma species within a mixed culture, or even on primary isolation plates. However, the finding that many immunodominant antigens

exposed on the mycoplasmal cell surface undergo rapid phase- and size-variation (see the section Antigenic Variation) puts severe restrictions on the use (as a reagent in colony immuno-blotting) of monoclonal antibodies directed to such a variable antigen (Rosengarten and Yogev, 1996). The use of a polyclonal antiserum is, therefore, preferable in these tests.

A great variety of diagnostic procedures based on detection of specific antibodies in sera of humans and animals infected with mycoplasmas have been devised; the procedures as well as the evaluation of the results are discussed in detail in the relevant chapters of *Molecular and Diagnostic Procedures in Mycoplasmology*, volume II (Tully and Razin, 1996) and in Waites et al. (2001).

Serodiagnosis consists of examining serum samples for antibodies that inhibit the growth and metabolism of the organism or form mycoplasmal antigen-antibody complexes that fix complement. The antibody response in mycoplasmal pneumonia is most easily demonstrated by complement fixation, reacting acute- and convalescent-phase sera with intact organisms or their lipid extract as antigen. Development of more defined and specific antigens based on immunodominant *M. pneumoniae* membrane proteins, such as P1 (Gerstenecker and Jacobs, 1993; Tuuminen et al., 2001) or the 116-kDa protein (Duffy et al., 1999) was attempted. A four-fold or greater antibody rise is considered indicative of recent infection, whereas a sustained high antibody titer may not be significant because a relatively high level of antibody may persist for at least 1 year after infection (Daxboeck et al., 2003). A variety of rapid tests based on indirect agglutination of erythrocytes or latex particles coated with *M. pneumoniae* antigens or on an immunofluorescence assay detecting IgM antibodies to *M. pneumoniae* (Dorigo-Zetsma et al., 1999) have been developed, and some are commercially available. A variety of rapid tests based on indirect agglutination of erythrocytes or latex particles coated with *M. pneumoniae* antigens, or an immunofluorescence assay detecting IgM antibodies to *M. pneumoniae* (Dorigo-Zetsma et al., 1999) have been developed, and some are commercially available. Enzyme immunoassay test kits presently represent the largest market share of commercial mycoplasma serologic tests (Suni et al., 2001; Tuuminen et al., 2001; Waites et al., 2001).

In summation, although PCR considerably extends the arsenal of reliable laboratory techniques, serology still retains importance in diagnosis of *M. pneumoniae* infections and of complications associated with *M. pneumoniae* infections. IgM antibodies are frequently diagnostic in children because primary disease is most likely to occur in children. In adults, on the contrary, IgM antibodies alone may not be diagnostic. Serology using IgA and IgA-antibody detection remains the method of choice for the diagnosis of acute infection in adults (Watkins-Riedel et al., 2001). A combination of *M. pneumoniae*-specific PCR in nasopharyngeal aspirates with an IgM-capture immunoassay in acute phase sera increases the sensitivity of laboratory diagnosis of *M. pneumoniae* and provides a laboratory result in only 1–2 days (Ferwerda et al., 2001).

Restriction Endonuclease Analysis

The classic tests for mycoplasma identification and classification have been supplemented by a variety of tests based on genomic DNA analysis. Restriction enzyme analysis of the mycoplasma genome provides a convenient and cost-effective means of determining DNA sequence variations among strains of mollicute species (Razin and Yogev, 1995). The method involves comparison of the number and size of fragments produced by digestion of the chromosomal DNA with a restriction endonuclease that cuts DNA at a fixed position within a specific recognition site, usually composed of 4–6 bp. Because of the high specificity of restriction endonucleases, complete digestion of a given DNA with a specific restriction endonuclease provides a reproducible array of fragments. Separation of the fragments by agarose gel electrophoresis and staining with ethidium bromide provides a restriction pattern that can be compared with that of related strains. Variations in the array of fragments generated by a specific restriction endonuclease are called "restriction fragment length polymorphisms" (RFLPs). These RFLPs can result from sequence rearrangements, insertion or deletion of DNA segments, or from base substitutions within the restriction endonuclease cleavage sites. Restriction endonuclease analysis (REA) has become a most useful taxonomic tool, facilitating the identification and classification of mycoplasmal isolates as well as providing means for evaluating the degree of genotypic heterogeneity of strains within established species (Razin, 1992a, b; Djordjevic et al., 2001). In addition, REA provides valuable information on the type and number of specific nucleotide sequences in the genome, serving as a basis for construction of physical genomic maps. The great advantage of chromosomal REA is that it is universally applicable and sensitive because the entire genome is evaluated for RFLP, and in addition, it is relatively easy to perform. Moreover, unlike sodium dodecyl sulfate (SDS)-PAGE of mycoplasmal cell proteins, REA is not susceptible to contamination by culture medium contaminants, and

requires very little (3–5 µg) of unlabeled DNA per test (Razin and Yogev, 1995).

A more recently developed technique, named "amplified-fragment length polymorphism" (AFLP) is a whole genome fingerprinting method based on the selective amplification of restriction fragments. This multistep procedure combines in an elegant manner the power of PCR with the informativeness of REA. AFLP yields more complex banding patterns than other DNA fingerprinting methods, increasing the discrimination between strains. In this way AFLP can be used as a device in studies of epidemiology, pathogenicity, and genetic variability in natural populations of mollicute species (Koktovic et al., 1999).

Southern blot analysis of genomic DNA (digested by a restriction enzyme and hybridized with a cloned conserved gene or a specific genomic fragment as a probe) has become a most useful tool in the identification, classification and subtyping of mollicutes (Razin, 1992; Yogev and Razin, 1995). The plasmid pMC5, carrying the entire 23S and 5S and most of the 16S rRNA genes of *M. capricolum* (Amikam et al., 1982) was among the first plasmids to be used as probes in Southern blot analysis of mycoplasmal DNA. Because restriction sites and flanking sequences differ within the rRNA operons of various mollicutes (Amikam et al., 1984; Razin et al., 1984), hybridization patterns peculiar to different mollicute species or strains are produced (Figs. 14 and 15). A restriction enzyme having a 6-bp recognition site will usually cut the one or two mycoplasmal rRNA operons in a few sites, so that the hybridization patterns are usually simple and much easier to compare than the multiband restriction patterns obtained by REA of entire genomic DNA. Southern blot hybridization with cloned rRNA genes as probes has been named "ribotyping." However, other conserved genes can be employed as probes, such as the *tuf* gene, encoding the elongation factor EF-Tu. The mollicute genome carries only one copy of this gene, so that the hybridization patterns obtained with restricted mollicute DNAs are also very simple and easy to compare (Razin, 1992).

Polymerase Chain Reaction

The introduction of PCR to diagnostics in the late 1980s pushed aside many of the previously developed DNA probes and commercial kits (Razin, 1994). The PCR tests are several orders of magnitude more sensitive than those based on direct hybridization with a DNA probe. Moreover, PCR is fast, copying a single DNA sequence over a billion times within three hours. Nucleic acid amplification techniques are not

Fig. 15. Hybridization patterns of DNAs from eight *Mycoplasma pneumoniae* strains collected from different epidemics. A) DNAs digested by *Hind*III or by *Eco*RI and hybridized with the rRNA gene probe pMC5. From Yogev et al. (1988a). B) DNAs of the same strains digested by *Cla*I and hybridized with the elongation factor *tufA* gene of *Escherichia coli*. From Yogev et al. (1988c).

Fig. 14. Southern blot hybridization of *Eco* RI-digested DNAs of various mycoplasmas (*Mycoplasma pneumoniae*, *M. genitalium*, *M. gallisepticum*, *Spiroplasma citri*, *M. pirum* and *Acholeplasma laidlawii*) with the rRNA gene probe pMC5, showing hybridization patterns peculiar to the different mollicute species. From Yogev et al. (1989).

limited by the ability of an organism to grow in culture, a feature of paramount importance considering the fastidious nature of the mycoplasmas and the fact that not all mollicutes (notably the phytoplasmas and the hemoplasmas) can be cultured in vitro. Thus, PCR-amplified 16S rDNA subjected to RFLP analysis has become the method of choice for routine detection, differentiation and classification of phytoplasmas (Marcone et al., 2000; Seemuller et al., 2002) and hemoplasmas (see the section Phylogeny and Taxonomy).

Detailed descriptions of methodology and evaluation of PCR-based tests in diagnostics can be found in the relevant chapters of *Molecular and Diagnostic Procedures in Mycoplasmology* (Tully and Razin, 1996b). The first major step in the development of a PCR-based test is the selection of appropriate target sequences for amplification. The sequence to be amplified can be chosen from a published mycoplasmal gene sequence or from a randomly cloned DNA fragment demonstrated to be specific for the detection of the mycoplasma (Caron et al., 2000; Kong et al., 2001). Complete or almost complete sequences of the 16S rRNA genes are now available for almost all the established mollicute species, and can be retrieved from databanks (see the section Phylogeny and Taxonomy). This information enables the selection of a variety of target sequences, starting with sequences in the highly conserved regions of the genes, producing primers of wide specificity (also known as "universal primers"). These primers will react with the DNA of any mycoplasma or even with the DNA of other prokaryotes and may be satisfactory for detection of mycoplasma infection in cell cultures, where the goal is just to screen the cultures for contamination. In case of a need to differentiate between contaminating mycoplasmas, a multiplex PCR system can be employed, consisting of a universal set of primers along with primer sets specific for mycoplasma species commonly infecting cell cultures (Choppa et al., 1998). The mycoplasmal 16S rRNA genes carry in addition to the conserved regions more specific variable regions, as well as specific 16-23S intergenic spacer regions (Tang et al., 2000; Kong et al., 2001). Primers can be selected from these regions with various degrees of specificity, ranging from clusters of species, single species, down to the subspecies level (Kong et al., 2001).

Specimen preparation for PCR testing is an important parameter that should be optimized. Because mycoplasmas have no cell walls, boiling of the sample, following its concentration by centrifugation, is often sufficient to make the organisms' DNA accessible. However, some clinical samples may contain undefined inhibitors of the PCR reaction, reducing the efficiency of amplification, so that DNA extraction has to be employed. Appropriate positive and negative controls should be included in each PCR test to rule out the presence of inhibitory substances. The methodology of target sequence amplification and optimization of the PCR test conditions, as well as the identification of the PCR products by electrophoretic analysis are detailed in De Barbeyrac and Bebear (1996). More recently, colorimetric procedures for identification of specifically amplified double-stranded DNA captured in wells of a microtiter plate were developed. These systems, some of which are commercially available as kits, are cost effective alternatives to gel electrophoresis, particularly for screening of moderately large sample numbers (Bashiruddin et al., 1999).

Although conventional PCR tests should under optimal conditions detect the DNA of a single mycoplasma, this rarely happens in testing clinical material. Two-step (nested) PCR has been devised to increase sensitivity to a level enabling detection of a single mycoplasma in a clinical sample. Another advantage of nested PCR is that the second-round PCR serves to confirm the specificity of the first-round PCR (Harasawa, 1996). Another approach is that termed "Random Amplified Polymorphic DNA (RAPD)," or "Arbitrary Primer PCR (AP-PCR)." It involves PCR amplification with a single arbitrary primer at low stringency, resulting in strain-specific arrays of DNA fragments that can reproducibly distinguish even closely related strains of a species. The simplicity of this procedure and the availability of commercial kits should facilitate the use of RAPD analysis as a routine procedure for identification of strains of a mollicute species, employing the RAPD patterns to investigate disease outbreaks for epidemiologic tracking (Cousin-Allery et al., 2000).

Numerous publications compare the results of the PCR technique with culture and serological testing for *M. pneumoniae* (for references, see Razin [2002] and Daxboeck et al. [2003]). Most publications indicate that PCR yields higher sensitivity and much faster results (for examples, see Ferwerda et al. [2001] and Templeton et al. [2003]). PCR assays can usually detect as little as a few femtograms (fg) of mycoplasmal DNA. When translated into numbers of organisms, 1 fg of mycoplasmal DNA is approximately equivalent to the genomic DNA of a single mycoplasma cell (i.e., about 1000 kb). The ability to detect by PCR a single mycoplasma cell makes PCR the most sensitive detection method available, even more sensitive than culture. In theory, a positive culture can be derived from a single mycoplasma cell. In practice, however, for many reasons

(including mycoplasma cell aggregates, multi-nuclear filamentous forms, and defective or nonviable cells) a positive mycoplasma culture requires an inoculum equivalent to about 100–1000 cells. This is known for other bacteria as well and is the reason for using the expression colony-forming units (cfus) or color-changing units (ccus) rather than number of cells per milliliter (Razin, 1994).

Obviously, PCR should be applied in the case of patients with an impaired immune response where serological tests may be much less effective. Positive PCR results for *M. pneumoniae* in culture-negative persons without evidence of respiratory disease suggests either inadequate specificity of the test, persistence of the organisms after infection, or their existence in asymptomatic carriers, making interpretation of such results difficult (Waites et al., 2001). In other words, does a sensitive technique such as PCR lead to 'over-diagnosis' of *M. pneumoniae* infections and is over-diagnosis partly due to detection of *M. pneumoniae* in healthy carriers? It is still difficult to answer these questions in a definitive manner.

In conclusion, PCR detection of mycoplasmas is still too labor-intensive, expensive and complex to be carried out routinely in most clinical microbiology laboratories. Development of satisfactory commercial PCR kits may simplify the procedures and bring about better standardization of the technique, and if available at a reasonable cost, PCR could become the major method for diagnosis of mycoplasmal infections.

Preservation of Cultures

Cultures of mycoplasmas may retain viability at 4°C for weeks, particularly when glucose is omitted from the medium or when bovine serum albumin and lipids replace serum (Taylor-Robinson and Behnke, 1987). However, prolonged storage of unfrozen cultures is unreliable, so that for long-term preservation, fast freezing of young cultures to –70°C (Raccach et al., 1975) or freeze-drying is recommended. Detailed procedures have been described and evaluated by Leach (1983).

The noncultivability of phytoplasmas so that their identification is based on molecular genetic analyses of their genomic DNA has led to the development of a method using microwave heat treatment of infected plant tissues to preserve phytoplasmal DNA in situ without compromising its stability (Wang and Hiruki, 1998). This treatment offers not only the preservation of phytoplasmal DNA, useful for molecular diagnosis, but also a way for safe international exchange of experimental materials without violating quarantine regulations.

Physiology

The Cell Membrane

MEMBRANE PROTEINS Lacking a cell wall and intracytoplasmic membranes, the mollicutes have only one type of membrane, the plasma membrane. The ease with which this membrane can be isolated and the ability to introduce controlled alterations in its composition have made mycoplasma membranes effective tools in membrane research (Razin, 1978, 1993).

Proteins constitute over two thirds of the mycoplasma membrane mass, the rest being membrane lipids. The structure and function of mycoplasmal membrane proteins have recently been reviewed by Wieslander and Rosen (2002). Membrane lipoproteins have attracted much attention in recent years as their relative abundance in mycoplasma membranes is most remarkable in contrast to the limited number of lipoproteins in membranes of other eubacteria. On the basis of characteristic lipoprotein-specific features, Himmelreich et al. (1996) annotated 46 putative lipoprotein genes in the *M. pneumoniae* genome, while 21 lipoprotein genes have been annotated in the *M. genitalium* genome (Fraser et al., 1995; Himmelreich et al., 1997). The 25 additional lipoprotein genes in *M. pneumoniae* are only gene amplifications, which are frequently organized in sequential order (Herrmann and Reiner, 1998). The *M. pulmonis* genome encodes 56 lipoproteins (Chambaud et al., 2001), while the *U. urealyticum* (*parvum*) genome appears to be the richest, encoding 74 lipoproteins (Glass et al., 2000). The unusually high number of lipoproteins in mollicutes may be attributed to the absence of a cell wall and a periplasmic space in these organisms. Mollicutes possess typical eubacterial signal peptides that direct the newly synthesized proteins into a secretory pathway for transport across the cell membrane (Yogev et al., 1991b). Still, the mollicute lipoprotein signal peptides are longer than for any other bacteria, a feature possibly associated with their thicker cholesterol-containing cell membrane, requiring longer transmembrane segments (Edman et al., 1999).

For surface-exposed membrane proteins which have to function on the cell outside, acylation of the proteins by long chain fatty acids is an effective way of anchoring the proteins to the cell surface. Cultivation of mollicutes (in the presence of labeled palmitate or myristate) labels a significant number of membrane proteins. Interestingly, membrane lipoproteins are

amongst the most dominant antigens in mollicutes (Razin et al., 1998; Wieslander and Rosen, 2002) and a majority of the mycoplasma cell surface antigens known to undergo antigenic variation are lipoproteins (see the section Antigenic Variation). At least some mycoplasmal membrane lipoproteins characterized in *M. pneumoniae*, *M. genitalium*, *M. fermentans* and *M. hyorhinis*, carry only two fatty acid acyl chains linked to the N-terminal cysteine residue via a thioester linkage between diacylglycerol and cysteine, lacking the third fatty acid linked to the amino group of cysteine, normally found in lipoproteins of other eubacteria. In fact, the transacylase responsible for acylation of the amino group of cysteine could not be identified in the *M. pneumoniae* and *M. genitalium* genomic analysis (Himmelreich et al., 1996, 1997; Razin et al., 1998; Calcutt et al., 1999; Le Henaff et al., 2001). Yet, chemical analysis of spiralin (the major lipoprotein of *S. melliferum*) showed that in addition to the two ester-linked acyl chains, a third acyl chain is amide-linked to the cysteine, resembling the classical eubacterial lipoproteins (Le Henaff and Fontenelle, 2000; Le Henaff et al., 2000). Similar findings were reported for the acylated lipoproteins of *M. agalactiae* (Le Henaff et al., 2000).

Molecular genetic methodology has recently enabled the preliminary characterization of membrane proteins of the uncultured phytoplasmas. The phytoplasma membrane proteins characterized thus far are apparently not lipoproteins. They appear to possess cleavable N-terminal signal sequences and a hydrophobic C-terminal anchor. The central hydrophilic domain is located on the outside of the cell membrane (Blomquist et al., 2001; Barbara et al., 2002).

MEMBRANE LIPIDS Virtually all mycoplasma lipids are located in the cell membrane and, as in other biological membranes, consist of phospholipids, glycolipids, and neutral lipids. A detailed description of the composition, distribution and biosynthesis of mycoplasmal membrane lipids can be found in Rottem and Kahane (1993). As mentioned in the Genome Sequencing section, the mycoplasmas are partially or totally incapable of fatty acid synthesis, and they depend on the host (or the culture medium) for their supply. In addition, most mycoplasmas require cholesterol for growth, a unique requirement among prokaryotes (Rottem, 2002b). The fatty acid residues of membrane phospholipids and glycolipids, as well as cholesterol, constitute a major portion of the hydrophobic core of the membrane. The dependence of mycoplasmas on the exogenous supply of fatty acids and cholesterol

has been one of their greatest advantages as models for membrane studies. The ability to introduce controlled alterations in mycoplasma membrane-lipid composition, simply by controlling the composition and content of fatty acids and sterols in the growth medium, has been used most effectively in elucidating the molecular organization and function of the lipids in mycoplasma membranes (Razin, 1993; Wieslander and Rosen, 2002).

Almost all recent studies concerning mycoplasma lipid biosynthesis and its regulation were carried out on *A. laidlawii*, an organism which has been used for a long time as a model organism, particularly in studies dealing with the physical state of the membrane (Razin, 1978, 1993). It has been the organism used by Wieslander, Rilfors, Lindblom and their associates at Umea University to show that altering the polar headgroup structure of membrane phospho- and glycolipids (and changing the acyl chain structure) are the two strategies employed by the organisms to adapt their membrane lipid composition to various environmental and physiological conditions (reviewed by Wieslander and Rosen [2002]). A common way in prokaryotes to respond to varying growth temperatures is to regulate the degree of unsaturation of the acyl chains of the lipids. *Acholeplasma laidlawii* cannot synthesize unsaturated fatty acids and can only synthesize limited amounts of saturated fatty acids when the organism is grown in a thoroughly lipid-depleted medium. Thus, at least one exogenous fatty acid must be supplied in the growth medium for the cells to grow.

Acholeplasma laidlawii exploits a different mechanism to cope with changes in environmental conditions, namely, altering the proportion of lipids with different polar head groups. The Umea group has hypothesized and experimentally supported that *A. laidlawii* strain A-EF22 regulates its membrane lipid composition to maintain a proper balance between lipids forming a lamellar crystalline phase, and lipids forming reversed nonlamellar phases. The first acholeplasmal lipid shown to form a nonlamellar phase was monoglucosyl diacylglycerol (MGlcDAG). The other major glycolipid, diglucosyl diacylglycerol (DGlcDAG), resembles the acholeplasmal phospholipids in forming solely the lamellar phase at all temperatures and with all acyl chain compositions. Then, acholeplasma were found to be capable of varying the proportion of the MGlcDAG and DGlcDAG in response to the prevailing growth conditions (Wieslander and Rosen, 2002).

In conclusion, the extensive studies of the Umea laboratory show that several basic features of the membrane homeostasis mechanism in *A. laidlawii* (including the maintenance of

phase equilibria, spontaneous curvature, and surface charge density of the membrane lipid bilayer) are dependent upon the physical properties of the bilayer. In addition, these features are sensed by the two consecutively acting glucosyltransferases synthesizing the major lipids MGlcDAG and DGlcDAG. Though the fine details of the molecular mechanisms regulating the synthesis of the two glucolipids have to be worked out, the experiments with the purified MGlcDAG synthase suggest that enzyme regulation may depend on the enzyme proteins themselves, not requiring a complex multiprotein regulatory mechanism (Berg et al., 2001; Wieslander and Rosen, 2002).

Metabolism

ENERGY-YIELDING PATHWAYS The small genome size of mollicutes precludes their possession of an extensive range of metabolic activities present in other bacterial groups. Demonstrated metabolic activities appear primarily to be associated with energy generation, rather than with the provision of substrates for synthetic pathways. All the mollicutes so far examined have truncated respiratory systems. They lack a complete tricarboxylic acid (TCA) cycle and have no quinones and cytochromes, ruling out oxidative phosphorylation as an ATP-generating mechanism (Pollack, 2002b). Thus, the demonstrated energy-yielding pathways of mollicutes produce low ATP yields and relatively large quantities of metabolic end products, depleting in some cases host tissues of the specific substrate metabolized.

On the basis of their ability to metabolize carbohydrates, the mollicutes are divided into fermentative and nonfermentative organisms. Members of the fermentative group produce acids from carbohydrates, decreasing the pH of the growth medium. Though fermentative mycoplasmas may differ in their ability to utilize sugars other than glucose, this property has only a limited taxonomic value. Interestingly, *M. fermentans* isolates from the urogenital tract of AIDS patients were found to use fructose in preference to glucose (Ozcan and Miles, 1999). In man, high fructose concentrations relative to glucose are associated only with seminal vesicle secretions. Thus, fructose-utilizing *M. fermentans* strains may be associated particularly with seminal vesicles and their secretions, and be transmitted in semen.

The sequencing projects of the *M. genitalium* and the *M. pneumoniae* genomes (Fraser et al., 1995; Himmelreich et al., 1996) showed that these mycoplasmas carry all the enzymes of the Embden-Meyerhof-Parnas pathway, whereas the second pathway for metabolizing glucose (the pentose phosphate shunt) is incomplete.

Pyruvate generated by glycolysis can be further metabolized either to lactate by lactate dehydrogenase, or to acetyl-CoA by the pyruvate dehydrogenase pathway. Most of the nonfermentative mollicutes, and some fermentative species as well, possess the arginine dihydrolase pathway. Arginine hydrolysis by this pathway results in the production of ornithine, ATP, CO_2 and ammonia, raising the pH of the culture medium (Razin, 1978). The pathway consists of three enzymes: arginine deiminase, ornithine carbamoyl transferase, and carbamate kinase. The degradation of arginine is coupled to equimolar generation of ATP by substrate-level phosphorylation. The role of this pathway as a sole energy-generating source in nonfermentative mollicutes has been frequently questioned (Razin, 1978; Himmelreich et al., 1996, 1997). The demonstration of an arginine-ornithine antiport system in *Spiroplasma melliferum* (requiring no ATP for arginine import into the cells) supports an energetic advantage in arginine utilization (Shirazi et al., 1995), but the question of whether arginine degradation can serve as a sole, or even a major, energy-generating mechanism remains unanswered.

The arginine dihydrolase pathway can be found also in some fermentative *Spiroplasma* and *Mycoplasma* species (see appendix in Tully and Razin [1996]). In this case, when both glucose and arginine are present in the medium, the acids produced by glycolysis would mask the alkalinization caused by the ammonia liberated on arginine degradation, hampering the test for arginine utilization by the simple pH change assay. Yet, as was shown in a nuclear magnetic resonance (^{13}C-NMR) study, the accumulation of lactate and breakdown of arginine by *M. fermentans* were observed in the simultaneous presence of both glucose and arginine, suggesting that glucose utilization has little or no effect on deamination of arginine to citrulline (Olson et al., 1993). Furthermore, proteome analysis of *S. melliferum* showed that the arginine dihydrolase enzyme proteins were constitutively expressed also in the presence of glucose (Cordwell et al., 1997a).

Some mycoplasmas, such as *M. agalactiae*, *M. bovigenitalium* and *M. bovis* (see appendix in Tully and Razin [1996]) metabolize neither sugars nor arginine but can oxidize organic acids (lactate and pyruvate) to acetate and CO_2 (Taylor et al., 1994). The contribution of the oxidation of these substrates to the energy provision of the above mycoplasmas should be worked out. Another potential energy-yielding mechanism in mollicutes is based on ATP generation from acetyl phosphate and adenosine 5′-diphosphate by acetate kinase, coupled with acetyl phosphate formation from acetyl coenzyme A (acetyl-CoA)

by phosphate acetyl transferase; both enzymes are commonly found in fermentative and non-fermentative mollicutes. Acetyl-CoA can be produced by oxidative phosphorylation of pyruvate by mycoplasmas (Razin, 1978). The weight that should be given to this pathway in energy metabolism of mollicutes has not been critically evaluated.

Energy metabolism of ureaplasmas presents a special case. Neither glycolysis nor arginine dihydrolase-ATP- or acetate kinase-ATP-generating pathways could be detected in these organisms. Ureaplasmas are unique among the mollicutes in possessing a very potent urease. Although protein and gene analysis of the ureaplasmal urease complex has shown its subunit structure and composition to resemble the other prokaryotic ureases, the specific activity of the ureaplasmal urease is much higher and was estimated to exceed that of jack bean urease by about 100 fold (Blanchard et al., 1988; Thirkell et al., 1989; Smith et al., 1993). More importantly, ureaplasmas appear to be unique among prokaryotes in requiring urea for growth (Razin, 1978). Thus, the specific urease inhibitor, flurofamide, inhibits ureaplasma growth (Blanchard et al., 1988). The dependence of ureaplasmas on urea for growth has led to the hypothesis that intracellular urea hydrolysis and the resulting intracellular accumulation of ammonia and ammonium ions is coupled to ATP synthesis through a chemiosmotic type of mechanism (Razin, 1978). Experimental support for the generation of a transmembrane potential, with resultant ATP synthesis through the ureaplasmal F_0F_1 ATPase, first provided by Romano et al. (1980), was later extended and confirmed by Smith et al. (1993). At an external pH of 6.0 (the pH optimum for ureaplasma growth in vitro), urea hydrolysis generated an ammonia chemical potential equivalent to almost 80 mV and, simultaneously, an increase in proton electrochemical potential (δp) of about 24 mV with resultant de novo synthesis of ATP (Smith et al., 1993). When the external pH of the growth medium reaches about 8.1, owing to ammonia released from the cells, the intracellular pH rises to 8.6 and urease activity ceases (as shown by pH activity profile of the ureaplasmal urease). Generation of ATP is blocked at this alkaline pH, leading to the abrupt and steep decline of growth, characterizing the ureaplasmal growth curve (Fig. 13). Inhibition of the urease by flurofamide abolished both the chemical potential and the increase of δp, such that ATP synthesis decreased to about 5% of the normally obtained levels (Smith et al., 1993; Glass et al., 2000). Lansoprazole, another specific urease inhibitor, also inhibited ATP synthesis and ureaplasma growth (Nogata et al., 1995). Note that the pH of the urogenital tract is usually on the acid side of neutrality, corresponding to the pH values optimal for ureaplasma growth, maximum increase in δp, maximum ammonia chemical potential, maximum urease activity, and maximum ATP generation.

Resembling other eubacteria, mollicutes possess an F-type ATPase (Razin, 1978). The number and order of genes of the *atp* operon coding for the F_0F_1 ATPase of *M. gallisepticum* (Rasmussen et al., 1992), *M. genitalium* (Fraser et al., 1995) and *M. pneumoniae* (Hilbert et al., 1996) are identical with those of *E. coli* and *Bacillus subtilis*. However, the b subunit (*atpF*) of the mycoplasmal ATPase was found to carry the characteristic features of a lipoprotein (Pyrowolakis et al., 1998). This feature, not known for any other eubacterial ATPase, may explain the long-known inability to detach the mycoplasmal ATPase activity from the cell membrane by varying the osmolarity of the medium or by treating the membrane with EDTA (Razin, 1978).

TRANSPORT The expectation to find in *M. genitalium* and *M. pneumoniae* a proportionally high number of genes involved in transport of the many essential nutrients required for growth has not been substantiated (Fraser et al., 1995; Himmelreich et al., 1996; Paulsen et al., 1998; see the section Genome Sequencing). This has led to the notion that at least some transport systems, such as those for amino acids and oligopeptides, might not be very specific, but little experimental evidence supporting this notion is presently available (Fraser et al., 2000; Pollack, 2002a).

Mycoplasmas, lacking both electron transport and a functional TCA cycle, generate ATP as their primary energy source, and consequently have mostly ATP-dependent permeases, whereas *B. subtilis* generates a proton motive force (*pmf*) as its primary source of energy and accordingly utilizes mostly pmf-dependent carriers (Paulsen et al., 1998). The ABC transporter systems are involved in import or export of a large variety of substrates, including sugars, peptides, proteins and toxins. In fact, the ABC transporters were the most frequent class of proteins found in *B. subtilis* (Kunst et al., 1997) and *E. coli* (Blattner et al., 1997). Generally, bacterial ABC importers have an ATP-binding domain and membrane-spanning domains located on separate polypeptides, whereas the ABC exporters may carry these components on the same or on different polypeptides (Blanchard et al., 1996). The first to show homology of mollicute proteins with ABC transporter systems were Dudler et al. (1988) studying *M. hyorhinis*. Putative ABC transporter genes were reported in *M. hyopneumoniae* (Blanchard et al., 1996), in *U. urealyticum* (*parvum*; Glass et al., 2000), *M. pulmonis* (Cham-

baud et al., 2001) and essentially in all the mollicute genomes sequenced so far; *M. pulmonis* showing the highest number, 56, of genes potentially encoding subunits for ABC transporters. The deduced amino acid sequence of these gene products showed significant homology with the ABC transporter proteins, particularly those of the eukaryotic multidrug resistance (MDR) protein family, a finding previously reported for other bacteria (Paulsen et al., 1998).

Mycoplasma genitalium and *M. pneumoniae* take up amino acids via two amino acid-polyamine choline (APC) transporters, and they probably accumulate peptides and polymers via two distinct ABC-type systems. These four permeases presumably provide the requisite precursors for protein synthesis (Himmelreich et al., 1996; Paulsen et al., 1998). Though trials to identify the substrate-binding domain (OppA) of the ABC oligopeptide transport in *M. genitalium* and *M. pneumoniae* failed (Himmelreich et al., 1996), the more recent study of Henrich et al. (1999) has identified in the homologous ABC transport system of *M. hominis* an OppA that can bind peptides of different length. This flexibility could endow the transporter with a less restrictive substrate specificity.

The highly efficient phosphoenolpyruvate-dependent sugar phosphotransferase transport systems (PTSs) have been reported in mollicutes long ago, and some of their components were partially characterized by classical biochemical methodology (Razin, 1978; Cirillo, 1993). Apparently, *M. genitalium* and *M. pneumoniae* divert a major fraction of their transport capacity to the task of sugar uptake for the purpose of carbon and energy acquisition. Thus, they possess both glucose and fructose phosphotransferase systems, two sugar permeases of the ABC superfamily, and a nonspecific channel protein of the major intrinsic protein (MIP) family, probably capable of transporting small, neutral, straight chain molecules, such as urea, glycerol and various polyols (Paulsen et al., 1998).

The recent molecular genetic approach has enabled a more precise and detailed description of the genes and protein components of the PTS systems (Zhu et al., 1998). Though the *M. genitalium* genome carries the genes for a PTS specific for glucose (Fraser et al., 1995), the closely related *M. pneumoniae* was found to carry two additional PTS systems, one with an apparent specificity for mannitol and another with an unknown specificity (Himmelreich et al., 1996, 1997). Fructose also appears to be imported by the PTS system of *M. pneumoniae*, though Himmelreich et al. (1996) failed to detect in the mycoplasma *fruF*, which is part of the fructose operon in enteric bacteria. Interestingly, the spiroplasmas that have the ability to multiply in insect hosts have, in addition to the glucose and fructose PTS genes, also the genes encoding for the trehalose-PTS enzyme II (trehalose being the major sugar of the insect hemolymph; Andre et al., 2003). A defective PTS system, lacking component E1, was found in *U. urealyticum* (*parvum*) possibly explaining the inability of this mycoplasma to utilize sugars (Glass et al., 2000).

Genetics

Genome Structure

The application of pulse-field gel electrophoresis (PFGE) to mycoplasma genome size determinations (Neimark and Lange, 1990) has provided a much more accurate and labor-saving procedure than the previously used renaturation kinetics method, resulting in a wealth of genome size data. The data show a continuum of genome sizes among mollicutes (less than 600 kb to over 2200 kb) with overlapping values between mollicute genera. Thus, genome sizes of *Mycoplasma* species range from 580 kb for *M. genitalium* to 1380 kb for *M. mycoides* subsp. *mycoides* LC, whereas for the helical *Spiroplasma* species genome size ranges from 780kb for *S. platyhelix* to 2220 kb for *S. ixodetis* (Table 1). The genome size of representative members of 12 major phylogenetic groups of the uncultured phytoplasmas (Marcone et al., 1999) also varied (530–1350 kb), showing considerable size variation within the same phylogenetic group and resembling in this respect the cultured mollicutes. The Bermuda grass white leaf phytoplasma chromosome of 530 kb is not only the smallest mollicute chromosome found to date, but also the smallest chromosome known for any living cell. The size of the *Eperythrozoon suis* genome (reclassified now as a mycoplasma; see the section Phylogeny and Taxonomy) is about 750kb and that of *Haemobartonella felis* (reclassified as *Mycoplasma haemofelis*) is about 1199kb (Berent and Messick, 2003), which is well within the size range of mollicute genomes (Messick et al., 2000).

Clearly, genome size can no longer be taken as the definitive taxonomic criterion, used previously to distinguish higher taxa in Mollicutes (Razin, 1992a, b). Yet, as a general rule, *Acholeplasma* and *Spiroplasma* species, considered phylogenetically as early mollicutes, have larger genome sizes than *Mycoplasma* and *Ureaplasma* species, considered to be phylogenetically more recent (see the sections Phylogeny and Taxonomy). This is in agreement with the notion that Mollicutes have evolved by degenerative or reductive evolution, accompanied by significant losses of genomic sequences (Woese, 1987).

The mycoplasma genome is characteristically low in G+C content. With very few exceptions, the G+C content of mycoplasma genomes is within the range 24–33 mol% (see appendix in Tully and Razin [1996]). The G+C distribution along the genome is uneven. Thus, while the average G+C content of the *M. genitalium* genome is 32 mol%, the G+C content of its rRNA genes is 44 mol%, and of its tRNA genes 52 mol% (Fraser et al., 1995). The *M. pneumoniae* genes for the P1 and ORF6 adhesins, and their repetitive sequences, exhibit a G+C content as high as 56 mol%, though on the other extreme, the origin of replication of this mycoplasma has a G+C content of only 26 mol%. These G+C contents can be compared to 40 mol% of the entire *M. pneumoniae* genome (Himmelreich et al., 1996, 1997). Consequently, many of the mycoplasmal intergenic regions are richer in A+T than the coding regions, reaching values as high as 80–90 mol% (Yogev et al., 1991a; Dybvig and Voelker, 1996). The variable G+C content of coding regions within the mycoplasmal genome has phylogenetic relevance, indicating (in the case of the rRNA and tRNA genes) their highly conserved nature and (in the case of the adhesin genes) their possible exogenous origin (see the section Adhesion to Host Cells).

As in other prokaryotes, some of the adenine and cytosine residues in mycoplasmal genomes may be methylated (Dybvig and Voelker, 1996). In many mycoplasmas, the adenine residues at the GATC site are methylated, but in others, cytosine residues are methylated. Of special interest is the exclusive methylation of the genomic cytosine residues of *S. monobiae* (MQ-1) when they are located 5′ to guanine (CpG), a methylation trait considered unique to eukaryotes (Renbaum et al., 1990).

Extrachromosomal Elements

Among the mollicutes, spiroplasmas and acholeplasmas are most frequently infected by a variety of viruses (phages), whereas very few viruses are known to infect *Mycoplasma* species (Zou et al., 1995; Voelker and Dybvig, 1998). The characteristics of mollicute viruses have been described and discussed in detail previously (Maniloff, 1992; Renaudin, 2002). The mollicute phage DNA genomes (4–40kb) may be either circular or linear, and single- or double-stranded (Dybvig and Voelker, 1996). Of the very few phages infecting *Mycoplasma* species, the most recent phage discovered is the lysogenic phage MAV1, infecting *M. arthritidis* (Voelker and Dybvig, 1999). The finding that this phage is associated with highly arthritogenic strains, and that experimental infection of low-virulence *M. arthritidis*

strains with this phage increases significantly their arthritogenicity, suggest that MAV1 carries a virulence-enhancing factor. Sequencing of the MAV1 linear double-stranded genome (15,644 bp) revealed 15 ORFs (Voelker and Dybvig, 1999). The deduced product of one of the genes, designated "vir," contained a classic prokaryotic lipoprotein signal sequence. If MAV1 encodes in fact a lipoprotein, it may be speculated that this lipoprotein is incorporated into the *M. arthritidis* cell membrane and in this way induces the conversion of the avirulent phenotype into a virulent one (see also the section Pathogenicity).

Plasmids were detected in *S. citri* and in *M. mycoides* subsp. *mycoides* (Dybvig and Voelker, 1996). Extrachromosomal DNA molecules, most prevalent in phytoplasmas of various phylogenetic clades have been postulated to represent plasmids, inasmuch as no virus particles could be observed by electron microscopy (Rekab et al., 1999). The almost universal occurrence of plasmids in phytoplasmas suggests a function for these molecules. The possibility that phytoplasma plasmids may encode genes associated with pathogenicity (Nishigawa et al., 2002) has been raised, but evidence to support this notion is still unavailable. The potential of the mollicute phages and plasmids to serve as cloning and shuttle vectors has been a major reason for interest in these elements (see the section Gene Transfer).

Genome Sequencing and Gene Annotation

Mycoplasmas, carrying the smallest genomes of self-replicating cells, were among the first microorganisms selected for the genome-sequencing projects. The complete sequence of the genome of *M. genitalium* was the second to be reported (Fraser et al., 1995) following closely the report on the complete sequence of the *Haemophilus influenzae* genome (Fleischmann et al., 1995). The remarkable power and cost-effectiveness of the new genome sequencing technology have already resulted in the elucidation of the complete genome sequences (by January 2003) of over 90 eubacteria and archeons (see http://www.tigr.org/tdb/mdb/{the website of The Institute for Genomic Research}). Included among these are the mollicutes *U. urealyticum* (*U. parvum*; Glass et al., 2000), *M. pulmonis* (Chambaud et al., 2001), *M. penetrans* (Sasaki et al., 2002) and *M. gallisepticum* (Papazisi et al., 2003). The genomes of *M. hyopneumoniae*, *M. mycoides* subsp. *mycoides*, and *S. citri* are at advanced or final stages of sequencing. A large part of the *S. kunkelii* genome has been sequenced and annotated (Bai and Hogenhout 2002). Partial sequencing and gene characteriza-

Table 4. Gross properties of sequenced mollicute and *Bacillus subtilis* genomes.

Property	*U. urealyticum*	*M. pneumoniae*	*M. genitalium*	*M. pulmonis*	*M. penetrans*	*B. subtilis*
No. of base pairs	751,719	816,394	580,074	963,879	1,358,633	4,214,814
G+C content (mol%)	25.5	40.0	32.0	26.6	25.7	43.5
No. of putative protein coding sequences (ORFs)	614	689	484	782	1,038	4,112
No. of ORFs with no functional prediction or database match	295	230	152	204	453	1,722

Abbreviation: ORF, open reading frame.
Data from Fraser et al. (1995), Himmelreich et al. (1996, 1997), Kunst et al. (1997), Dandekar et al. (2000), Glass et al. (2000), Chambaud et al. (2001), and Sasaki et al. (2002).

tion of a phytoplasmal genome have recently been published (Melamed et al., 2003).

The voluminous genetic data provided by the genomic projects has opened the way for "comparative genomics" by which the total genomic complements of organisms could be compared. This provides an opportunity to explore the functional content of genomes and evolutionary relationships between them at a new qualitative level. Typically, about 40–60% of the genes of a newly sequenced bacterial genome display detectable similarity to protein sequences whose function is at least tentatively known. Gene density is consistent across many bacterial species, with about one gene per kilobase (Fraser et al., 2000). A detailed comparative genomic analysis of *M. genitalium* and its close relative *M. pneumoniae*, compared to that of *H. influenzae* and other eubacteria, was presented in Razin et al. (1998). Table 4 summarizes some of the gross properties of the mollicute genomes sequenced so far, in comparison with the *Bacillus subtilis* genome.

Examination of the mycoplasmal genomic data indicates the biochemical pathways where gene reductions took place, and helps us define the genes that are really essential for a minimal self-replicating cell. In fact, defining the minimal gene set required by a self-replicating cell has become a most attractive research subject, applying *M. genitalium* as a prime candidate for this purpose (Razin et al., 1998; Koonin, 2000; Hutchison and Montague, 2002). Moreover, Hutchison et al. (1999) have promoted the idea of constructing an artificial life form based on the genes found essential for *M. genitalium* survival following global transposon mutagenesis. The essential genes were defined, in part, by the absence of viable mutants containing a transposon insertion within a given coding region. Hutchison et al. identified in this way 243 *M. genitalium* genes whose functions are not essential for cell viability. From this analysis (coupled with statistical and computational analysis), the authors concluded that 265–350 of the 517 genes in *M. genitalium* are essential for its life under

laboratory growth conditions, that is, in the presence of a full complement of essential nutrients and in the absence of environmental stress. Recent announcements in public communication media and in the journals *Nature* (Check, 2002) and *Science* (Zimmer, 2003) indicate that Craig Venter, Hamilton Smith, and Clyde Hutchison have already initiated a most ambitious endeavor directed at the synthesis of an artificial minimal cell. Their idea is to synthesize first an artificial genome, based on the genes found essential for life of *M. genitalium*, and to introduce this synthetic genome into *M. genitalium* cells from which the natural genome was removed. Whether the synthetic genome will hijack the cellular machinery of the genome-depleted host cell is, of course, a great mystery. A most interesting article relevant to the issue of the 'minimal cell' concept is that of Kobayashi et al. (2003). By systematic inactivation of *B. subtilis* genes, they could show that only 271 of the 4112 genes of this bacterium (Table 4) appear essential for its survival and growth under optimal conditions. Again, demonstrating how little genetic information is theoretically needed for the assembly of a minimal cell.

Genomic analysis of *M. genitalium* and *M. pneumoniae* has revealed the scarcity of genes involved in biosynthetic pathways (Fig. 16; for detailed discussion, see Razin et al. [1998] and Dandekar et al. [2002]). The two mycoplasmas have apparently lost during their reductive evolution all the genes involved in amino acid biosynthesis, as well as most of the genes involved in cofactor (vitamin) biosynthesis, so that to cultivate these mycoplasmas in vitro the medium has to be supplemented with the essential amino acids and vitamins. Very significant savings in genetic information have resulted through the loss of the cell wall during mycoplasma evolution. Significant gene savings are also pronounced in lipid metabolism. Most mycoplasmas cannot synthesize any fatty acids and depend, therefore, on the host for their supply (see the section Membrane Lipids). Being deficient in the ability to regulate membrane fluidity by prefer-
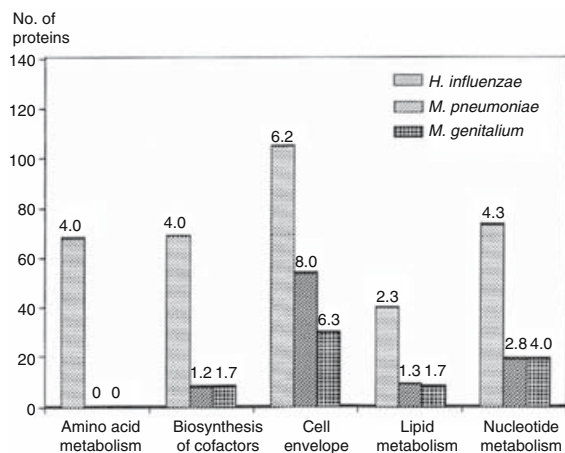
Fig. 16. Biosynthetic pathways genes in genomes of *Haemophilus influenzae*, *Mycoplasma pneumoniae* and *M. genitalium*. Numbers above the bars indicate the percentages of the total putatively identified genes. Based on data from Fleischmann et al. (1995), Fraser et al. (1995), and Himmelreich et al. (1996). From Razin et al. (1998).

Fig. 17. Cellular processes, metabolic pathways, and regulatory function genes in the genomes of *Haemophilus influenzae*, *Mycoplasma pneumoniae* and *M. genitalium*. Numbers above the bars indicate the percentages of total putatively identified genes. Based on data from Fleischmann et al. (1995), Fraser et al. (1995), and Himmelreich et al. (1996). From Razin et al. (1998).

ential fatty acid biosynthesis, most mycoplasmas overcome this deficiency by incorporating large quantities of exogenous cholesterol into their membrane. Cholesterol serves as a very effective buffer of membrane fluidity (Razin, 1978). Our early studies on mycoplasma nutrition revealed their requirement for the nucleic acid precursors, purines and pyrimidines. These may be provided by RNA and DNA degraded by the potent mycoplasmal nucleases (Razin, 1978). The present genetic data provide an explanation for these observations by revealing the scarcity in mycoplasmas of genes, and consequently of enzymes, responsible for purine and pyrimidine synthesis, while genes for salvage pathways utilizing purines and pyrimidines for the synthesis of ribonucleotides and their conversion to deoxyribonucleotides have been detected in mycoplasmas (Tham et al. 1993; Fraser et al., 1995; Himmelreich et al., 1996). Notably, the picture of extreme reduction in biosynthetic genes, described above for *M. genitalium* and *M. pneumoniae*, is less pronounced on analysis of larger mollicute genomes. Thus, the 1600kb genome of *S. kunkelii* carries genes for de novo synthesis of several amino acids and nucleosides, as well as regulatory factors not found in *M. genitalium* and *M. pneumoniae* (Bai and Hogenhout, 2002).

The number of genes involved in cellular processes, such as the *fts* genes associated with cell division, heat shock proteins, and genes for chaperones functioning in protein secretion, is definitely smaller in the mycoplasmas as compared to other eubacteria (Fig. 17). However, the major chaperone families HSP70 (DnaK), HSP60 (GroEL), and associated components (e.g.,

DnaJ, GroES, and GrpE) as well as the folding isomerase, and the trigger factor are present in *M. pneumoniae* and *M. genitalium*. In essence, all the constituents of a basic protein folding machinery are present (Bang et al., 2000). Yet, the genes for the chaperonins GroES and GroEL were not detected in *U. urealyticum* (*parvum*) so that different mollicute systems may vary in detail (Glass et al., 2000). The protein secretion system in *M. pneumoniae* is much less complex than in *E. coli*. The channel-forming proteins SecG, SecF, SecE, SecD were not identified in the mycoplasma, leaving only SecA, SecB, SecY, trigger factor, bacterial signal recognition particle, and FtszY as the components of a simplified translocon in *M. pneumoniae* (Himmelreich et al., 1996; Edman et al., 1999; Dandekar et al., 2000).

Many of the regulatory system genes found in other bacteria, such as the two-component signal transduction systems consisting of a sensor and response regulator, were not detected in the two mycoplasmas, but were present in *H. influenzae* (Fig. 17). *Mycoplasma genitalium* and *M. pneumoniae* lack recognizable vestiges of the histidine protein kinases or their target response regulators. Generally, a rough correlation can be observed between genome size and the number of putative protein kinases and protein phosphatases. Thus, while *M. genitalium* carries a single potential protein kinase, *Synechocystis* carries nine potential protein kinases and ten potential protein phosphatases (Shi et al., 1998).

Synechocystis (with its ability to survive on the barest of environmental resources, light and air) presumably has developed and maintained a more extensive cellular sensory, command and control apparatus to support its lifestyle than that of organisms (such as *M. genitalium* and *M. pneumoniae*) specialized to efficiently exploit more monotonous environmental niches (Shi et al., 1998). The *M. penetrans* genome differs from the other mycoplasma genomes sequenced so far (Table 3) by carrying the genes for a predicted two-component signal system (Sasaki et al., 2002). The absence of identifiable transcription factors from *M. genitalium* is most striking. While *E. coli* has 55 known transcriptional activators and 58 repressors, *M. genitalium*, apart from *nusA* and *nusG*, contains only a single transcription elongation factor (Ouzounis et al., 1996), and the *M. pulmonis* genome was found to carry only two transcription regulation factors (Chambaud et al., 2001). Relevant to this issue is the observation that FruR, the product of the *S. citri* gene *fruR*, acts as an activator of the fructose operon transcription in the spiroplasma. This appears to be the first mollicute operon for which regulation of transcription is documented (Gaurivaud et al., 2000c).

The genomes of *M. genitalium*, *M.* pneumoniae, and other mollicutes are deficient in genes coding for components of intermediary and energy metabolism (Fig. 17). Thus, the two mycoplasmas depend mostly on glycolysis as a means for synthesizing ATP. Genes that encode the components of the pyruvate dehydrogenase complex, phosphotransacetylase and acetate kinase, as well as a deficient pentose phosphate pathway were also detected (Fraser et al., 1995; Himmelreich et al., 1996). Most striking is the lack of many energy-yielding systems from the mycoplasmas. No TCA cycle and no quinones and cytochromes were found in any of the mycoplasmas tested (see the sections Energy-Yielding Pathways and Transport). The electron transport system in mycoplasmas is flavin terminated. Thus, ATP is produced in mycoplasmas by substrate-level phosphorylation, a less efficient mechanism than oxidative phosphorylation. In terms of evolution strategy, mollicutes may have concentrated two different functions in a single gene during genome size reduction. This consolidation is evidenced by the malate-lactate dehydrogenase (MDH and LDH) example (Cordwell et al., 1997b) where the gene putatively assigned to lactate dehydrogenase in *M. genitalium* and *M. pneumoniae* fulfills both LDH and MDH functions. A *Spiroplasma citri* gene was also found to encode a bifunctional protein, whose N-terminal domain has a ribosomal function, and the C-terminal domain is involved in inverted repeat sequence (IRS) binding and might have a

regulatory function at the IRS of the genomic DNA (Le Dantec et al., 1998). Along this line, a lipoprotein of *M. hominis* (functioning as an adhesin) was recently shown to function also as the substrate-binding domain OppA of an oligopeptide permease of *M. hominis* (Henrich et al., 1999). The inability to detect in the mollicute genomes sequenced so far the genes for nucleoside-diphosphate reductase and nucleoside diphosphate kinase was surprising, as these are considered indispensable, ubiquitous and highly conserved genes, playing a key role in the synthesis of nucleotide triphosphates. A way out of this problem was proposed by Pollack et al. (2002) suggesting that glycolytic kinases are capable of using a variety of purine and pyrimidine di- and trinucleosides as phosphate donors or acceptors rather than ATP, and in this way supply the required di- and tri-nucleotides.

Being dependent on the exogeous supply of many nutrients would predict the need of mycoplasmas for many transport systems. As can be seen in Fig. 17, the percentage of genes devoted to transport in *M. pneumoniae* and *M. genitalium* is not higher than in *H. influenzae*, though their absolute number is much lower. The small number of transport genes in the two mycoplasmas is even more striking when compared to the 281 transport and binding proteins annotated in *E. coli* K-12 (Blattner et al., 1997) and almost 400 in *B. subtilis* (Kunst et al., 1997). The apparent low substrate specificity of some of the mycoplasmal transport systems, such as those for amino acids (Himmelreich et al., 1996), may contribute to the significant gene saving observed in this category (see the section Transport).

The essential role of the basic processes of DNA replication, transcription and translation, leads to the expectation that saving of genes in these categories will be more restricted as compared to metabolic processes. Figure 18 shows that while the absolute number of genes involved in DNA replication in the mycoplasmas is still much smaller than in *Haemophilus*, the percentage of genes devoted to DNA replication and degradation in *M. genitalium* and *M. pneumoniae* is higher than in *H. influenzae*. This observation points to the essential biological role of these genes, so that evolutionary deletion of genes in this category had to be limited.

Obviously, defining the genes missing from the mycoplasmas is still of interest, as these are apparently of secondary importance. For example, the estimated number of genes functioning in DNA repair in *E. coli* is estimated to be about 100, and approximately 30 in *H. influenzae* (Fraser et al., 1995; Himmelreich et al., 1996; Blattner et al., 1997). Only 13 of the genes known to be involved in excision repair, recombination, and SOS repair of DNA were found

No. of
proteins

Fig. 18. DNA replication, transcription and translation genes in the genomes of *Haemophilus influenzae*, *Mycoplasma pneumoniae* and *M. genitalium*. Numbers above the bars indicate the percentages of total putatively identified genes. Based on data from Fleischmann et al. (1995), Fraser et al. (1995), and Himmelreich et al. (1996). From Razin et al. (1998).

genes involved in translation and in ribosome and tRNA synthesis is reflected in their G+C content. Thus, although the overall G+C content of the *M. genitalium* genome is 32 mol%, the G+C of the mycoplasmal rRNA and tRNA genes is 44 mol% and 52 mol%, respectively (Fraser et al., 1995). The degree of homology of the mycoplasmal ribosomal proteins to those of other eubacteria is also very high. Thus, the composition of mycoplasmal translation components resembles very much that of other bacteria. A marked difference can, nevertheless, be observed in the copy number of the rRNA and tRNA genes. Although *E. coli* and *H. influenzae* genomes carry seven and six copies of rRNA operons, respectively, *M. genitalium* and *M. pneumoniae* have only one copy (Table 5). Savings in the number of rRNA genes was long ago shown to be a general property of mycoplasmas, as the maximum copy number of rRNA genes in these organisms is only two, with one exception of three in *Mesoplasma lactucae* (Amikam et al., 1984; Bove, 1993). The number of tRNA genes in mycoplasmas is also kept to a minimum, with very few, if any, gene duplicates (Table 5). Thus, the *M. genitalium* and *M. pneumoniae* genomes carry only 33 genes (Fraser et al., 1995; Himmelreich et al., 1996), and *M. capricolum* carries 30 genes (Muto et al., 1990). These numbers compare with 54 in *H. influenzae* and 86 in *E. coli* (Fleischmann et al., 1995; Fraser et al., 1995; Blattner et al., 1997). Accordingly, the number of anticodons in the mycoplasmas is not much higher than in mitochondria and is close to the essential minimum for translation of all the amino acid codons by wobbling (Razin et al., 1998). Thus, the mycoplasmas appear to possess the minimum machinery needed for protein synthesis. Consequently, protein synthesis and cell replication in mycoplasmas is much slower than in *E. coli*. Thus, the velocity of the DNA replication fork was about 10 times slower in *M. capricolum* than in *E. coli* (Seto and Miyata, 1998). Yet, mycoplasmas appear to grow well in the protective and constant environment of their host and a slow replication rate cannot really be considered as a deficiency of a parasite that can only lose by killing its host. In fact, mycoplasma infections are usually rather mild and chronic in nature (see the section Pathogenicity).

There can be little doubt that the considerable gene number reduction depends primarily on the obligate parasitic lifestyle of mycoplasmas. Some genomic price has to be paid for parasitism; that is, mycoplasma cells must possess surface components enabling their attachment to the host cells. In some cases, including *M. genitalium* and *M. pneumoniae*, the mycoplasmas developed special attachment organelles. Obviously, significant numbers of genes are involved

by Himmelreich et al. (1996) in *M. pneumoniae*. Presumably, those genes detected in the mycoplasmas, including the uracil DNA glycosylase gene, the ABC excinuclease genes, and *recA*, must represent the genes really essential for DNA repair (see the section DNA Replication and Repair).

As to transcription, the percentage of genes devoted to transcription also is higher in the mycoplasmas than in *Haemophilus*, though their absolute number is definitely smaller in the mycoplasma (Fig. 18). Included in this category are the genes for the RNA polymerase subunits, which are basically similar in mycoplasma and the other eubacteria (see the section Transcription), and the genes for RNA helicases. The two mycoplasmas encode only a single σ factor, compared to at least 6 in *E. coli* (Blattner et al., 1997) and 18 in *B. subtilis* (Kunst et al., 1997). The *M. genitalium* genome carries only 2 ribonuclease genes, compared to 10 in *H. influenzae* (Fraser et al., 1995; Himmelreich et al., 1996).

The translation and protein synthesis machinery constitutes apparently the most conserved system in eubacteria, indicating that it represents a very efficient and successful evolutionary development. The highly conserved nature of the

Table 5. Ribosomal RNA operons, transfer RNAs, and UGA codon usage in mollicutes and other eubacteria.

Property	Mollicutes	Other eubacteria	References
No. of rRNA operons	1–2	1–10	Amikam et al., 1984
5S rRNA length	104–113 nucleotides	>114 nucleotides	Rogers et al., 1985
No. of tRNA genes	30 (*M. capricolum*)	84 (*B. subtilis*)	Himmelreich et al., 1996
	29 (*M. pulmonis*)	86 (*E. coli*)	Kunst et al., 1997
	33 (*M. pneumoniae*)		Blattner et al., 1997
	33 (*U. urealyticum parvum*)		Glass et al., 2000
			Chambaud et al., 2001
			Muto and Ushida, 2002
UGA codon usage	Tryptophan codon in *Mycoplasma*, *Ureaplasma*, *Spiroplasma*, *Mesoplasma*	Stop codon	Osawa et al., 1992
	Stop codon in *Acholeplasma* and *Phytoplasma*		

Adapted from Razin et al. (1998).

in construction of such an organelle (Balish and Krause, 2002). Some of these genes have already been identified and characterized (see the section Adhesion to Host Cells), but some have not and are apparently included in an as yet unidentified part of the mycoplasmal coding regions or genes.

TRANSCRIPTOMES AND PROTEOMES Experimental identification and confirmation of the proposed gene products at the cellular level has become complementary to identification by sequence comparison. Gene expression can be studied either at the level of transcription or at the level of translation. When these analyses are carried out on intact cells, the terms "transcriptome" (defined as the sum of all transcripts derived from a genome) and "proteome" (the expressed total protein complement of a genome) apply.

Transcriptome analysis uses microarray technology to determine expression level of thousands of genes all at once (Weiner et al., 2002a). For this purpose total RNA is isolated from the mycoplasma grown under defined laboratory conditions. The RNA is transcribed into a single-stranded complementary DNA and is labeled with [33]P. Specific probes are synthesized for all proposed ORFs by PCR, and the probes are immobilized on nylon membranes and tested for cross-hybridization with the labeled cDNA. This technique was used to analyze gene expression in *M. pneumoniae* grown under various conditions, such as heat shock, oxidative stress, and growth in the presence of different antibiotics (Weiner et al., 2000, 2002, 2003). Of the 688 proposed *M. pneumoniae* genes, about 600 were transcribed, but transcription of many genes was temperature dependent and therefore regulated, while numerous genes were constitutively expressed at all temperatures. Interestingly, a substantial proportion of genes with high transcription signals had no known function. Yet, their high expression level suggests that they code for important products (Weiner et al., 2002).

Proteome analysis involves 2D-gel electrophoresis for the separation of proteins according to isoelectric point (pI) and molecular mass, knowledge of the sequences of all cell proteins, and mass spectrometry for the characterization of individual proteins. The combination of peptide mass fingerprinting and peptide fragmentation (which matches the masses of in-gel proteolytically generated peptides against theoretically digested proteins from the database) has proven to be very effective and reliable (Regula et al., 2000; Weiner et al., 2002; Ueberle et al., 2002).

Mycoplasmas are particularly suitable for proteome analysis, as a high percentage of their proteins can be visualized by 2D-gel electrophoresis, analyzed and identified. Moreover, the highly conserved nature of a large part of mycoplasmal proteins facilitates their identification. The major limitations of the proteome approach are: 1) Not all cell proteins are expressed at a given time. Thus, visualizing an entire proteome under a specific set of experimental conditions is improbable. 2) The detection threshold of 2D-PAGE is a limiting factor, as a low copy number of protein molecules may not be detected. 3) Proteins with an isoelectric point higher than 11 cannot be separated well in the first dimension of immobilized pH gradients as well as proteins with 5 or more transmembrane segments. 4) Presently, proteome analysis is only useful for identifying proteins for which the DNA sequence of their genes is available. 5) Co- and posttranslational modification of proteins may increase the number of protein spots per gene (Ueberle et al., 2002).

The pioneering work on mollicute proteome analysis was carried out by Humphery-Smith and his colleagues on *Spiroplasma melliferum* (Cordwell et al., 1997a) and on *M. genitalium* (Wasinger et al., 1995) at a time when complete genome sequences were not available and the sensitivity and accuracy of mass spectrometry methods were inferior to those of today. In a more recent study, Wasinger et al. (2000) could resolve 427 protein spots in proteome analysis of exponentially grown *M. genitalium* cells. Of the 201 proteins of sufficient abundance, 158 could be identified by peptide mass fingerprinting. A reduction of 42% in protein spots was noticed in postexponentially grown cells. On the whole, only about one-third of the proteins in the predicted *M. genitalium* proteome could be identified by them. Proteome analysis of *M. pneumoniae* by Regula et al. (2000) characterized about 350 protein spots. These 350 proteins could be assigned to 224 genes. Clearly, the *M. pneumoniae* proteome map is still far from being as complete as that of *M. genitalium*. Ueberle et al. (2002) concluded that because of the technical problems discussed above, a complete proteome analysis based on the separation of cell proteins by 2D-gel electrophoresis cannot be achieved at the present, even for simple organisms like *M. genitalium* and *M. pneumoniae*.

DNA Replication and Repair

The recent mycoplasma genome sequencing projects, as well as the study of Barnes et al. (1994) on *M. pulmonis* DNA polymerases, have clarified considerably the nature of the mycoplasmal DNA polymerases. Apparently, the mycoplasmas carry the central enzyme for DNA replication—the DNA polymerase III holoenzyme (Pol III) that has the essential characteristics of the eubacterial Gram-positive Pol III. Although the genes responsible for building the major subunits of the holoenzyme, such as the α (*dnaE* or *polC*) and β subunits (*dnaN*) were detected, several other genes responsible for other subunits were missing from the *M. genitalium* and *M. pneumoniae* genomic analysis (Fraser et al., 1995; Himmelreich et al., 1996), indicating a simplified DNA replication complex in the mycoplasmas compared with that of *E. coli* or *H. influenzae*. Yet, most importantly, the mycoplasmal Pol III α-subunit (about 1400 amino acids) included the motif for 3′→5′ exonuclease activity typical for Pol C of Gram-positive bacteria (Barnes et al., 1994; Himmelreich et al., 1996; Kunst et al., 1997), providing an answer to the question of how DNA proofreading is done in mycoplasmas.

Barnes et al. (1994) presented experimental evidence for a second enzyme with DNA polymerase activity in *M. pulmonis*. This correlates with sequence data for *M. genitalium* and *M. pneumoniae*, indicating that at least these three *Mycoplasma* species carry two DNA polymerase genes. One codes for the larger protein (about 166kDa) with a 3′→5′ exonuclease activity and high sequence similarity to the *B. subtilis* polymerase III. The other gene codes for a Pol III homologue (about 100kDa) resembling more the *polC* gene from *E. coli* and lacking a 3′→5′ exonuclease domain. This smaller enzyme may correspond to the DNA polymerases lacking exonuclease activity isolated earlier from *M. orale* and *M. hyorhinis* (reviewed in Maurel et al. [1989]). The picture appears different for the recently sequenced genome of *Spiroplasma kunkelii* where its larger genome carries a *polA* gene that may encode the full length DNA polymerase I protein, including the proofreading and Klenow domains, resembling in this respect *Streptococcus pneumoniae* (Bai and Hogenhout, 2002).

Notably, though the major components of the DNA polymerase machinery are present in *M. genitalium* and *M. pneumoniae*, their genetic maps reveal the absence of many genes (including some major ones) involved in DNA replication (e.g., initiation, elongation, and termination of replication; Fig. 18). Most puzzling was the early failure to detect RNase H in the *M. genitalium* and *M. pneumoniae* genomes. This enzyme cleaves RNA in RNA-DNA hybrid molecules and is thought to be present in all living organisms. More recent reanalysis of the genomic data by Bellgard and Gojobori (1999) has identified new ORFs in both genomes. The most significant identification was that of RNase H, indicating the need for detailed reanalysis of genomic sequences by a more robust systematic approach for ORF identification.

Topoisomerases (topos) catalyze the interconversions of topological isomers of DNA molecules, fulfilling an essential role in DNA replication, transcription, recombination and repair. The mycoplasmal DNA topos, characterized so far, are the DNA gyrase and topoisomerase IV, both belonging to type II topos. Both mycoplasmal enzymes resemble their eubacterial counterparts in having a tetrameric structure composed of two subunits: A and B in the case of DNA gyrase, and the products of *parC* and *parE* genes in the case of topoisomerase IV (Ladefoged and Christiansen, 1994; Bebear et al., 1998a). The DNA gyrase introduces negative supercoils into a relaxed closed circular DNA molecule in a reaction requiring ATP. Interest in the molecular characterization of mycoplasmal topoisomerases has been boosted by the fact that these enzymes are the targets of fluoroquinolones, among the most

effective antimycoplasmal drugs (Kenny et al., 1999; Bebear and Bebear, 2002; Reinhardt et al., 2002; Bebear et al., 2003).

The three genes coding for the ABC excinucleases are present in *M. genitalium*, *M. pneumoniae* and *M. capricolum* so that together with Pol I, helicase II and ligase should provide the mechanism for repair of ultraviolet (UV) damage, such as crosslinking. While dark reactivation of UV damage (excision repair) operates in the above mycoplasmas, photoreactivation may be missing, as the photolyase (*phr*) gene could not be identified in *M. genitalium* and *M. pneumoniae*. The RecA protein plays a central role in recombination-repair, homologous recombination, and initiation of the SOS response. The *recA* gene has been found essentially in all the mollicutes analyzed so far (Zou and Dybvig, 2002). Surprisingly, *S. citri* R8A2 was found to lack a significant portion of the N-terminus of RecA (Marais et al., 1996) affecting its function. However, truncation of RecA is not a characteristic of other *Spiroplasma* species, such as *S. melliferum* (Cordwell et al., 1997a; Melcher and Fletcher, 1999). Not surprisingly, searching of DNA repair systems in mollicutes has been associated with the high mutation rates and high tempo of evolution in Mollicutes (Rocha and Blanchard, 2002). For an in-depth treatment of the issue of DNA replication and repair in mycoplasmas, the reader is referred to Zou and Dybvig (2002).

Transcription and Translation

The DNA-dependent RNA polymerase of mollicutes resembles in subunit structure that of other eubacteria. Thus, the core RNA polymerase of *M. genitalium* (Fraser et al., 1995) and *M. pneumoniae* (Himmelreich et al., 1996) is coded by the conserved genes *rpoA* α subunit, *rpoB* β subunit and *rpoC* β′ subunit. One of the peculiar properties of mollicutes is their resistance to the antibiotics rifamycin and streptolydigin (Bove, 1993; Table 5). Cloning and sequencing of the *rpoB* gene from *S. citri* (Gaurivaud et al., 1996; Laigret et al., 1996) and *M. gallisepticum* suggest that minor differences in amino acid sequences in the β-subunit Rif region, the region responsible for rifamycin binding, may confer rifamycin resistance.

Modulation of promoter selectivity of RNA polymerase by replacement of the σ subunits is an efficient way to alter the global pattern of gene expression in response to changes in environmental conditions. Thus, the *E. coli* genome carries at least 6 σ factors, and that of *B. subtilis*, 18 (Kunst et al., 1997). The presence of only one σ factor in the mycoplasmas (Himmelreich et al., 1996) suggests that the response to external stimuli in these organisms is not controlled by the level of expression of alternative σ factors. Mycoplasmas have conserved the heat shock response, and heat shock proteins (resembling the eubacterial DnaK [HSP70] and GroEL proteins) have been identified in several mollicutes. The *M. genitalium* and *M. pneumoniae* genomes carry genes for seven heat shock proteins (Fraser et al., 1995; Himmelreich et al., 1996). Almost as striking as the absence of a cell wall in these mycoplasmas is their lack of alternative σ factors, two-component signal transduction systems, or other means of gene regulation common to prototypical walled bacteria.

TRANSCRIPTION SIGNALS The mycoplasmal transcription signals resemble generally the classical eubacterial ones. The mycoplasmal –10 (Pribnow box) and to a lesser extent the –35 regions (consensus sequences) resemble the eubacterial promoter consensus sequences recognized by the RNA polymerase holoenzyme (Weiner et al., 2000). A consensus Shine-Dalgarno (SD) sequence could also be identified upstream to the initiation codon (Bove, 1993; Dybvig and Voelker, 1996) though in many cases the SD sequence could not be identified, indicating that signals other than the SD sequence may function as a ribosomal binding site for some mollicute genes. Termination of transcription in mollicutes appears to be independent of the termination factor Rho, as its gene could not be detected in the mycoplasmal genomes analyzed thus far.

RIBOSOMAL PROTEINS AND RIBOSOMAL RNAS The translation machinery of mycoplasmas is rather comprehensive. In *M. pneumoniae* and *M. genitalium*, it is comprised of 99 proposed genes, constituting about 15–20% of the genome's ORFs (Fig. 18). The mycoplasmal ribosomes are typically prokaryotic in size, shape and composition. The *M. pneumoniae* ribosomal protein genes are organized in operons, retaining some of the gene order found in the *E. coli* and *B. subtilis* genomes (Hilbert et al., 1996). Of the 50 ribosomal proteins found in *E. coli*, only the gene for protein S1 could not be detected in *M. genitalium* and *M. pneumoniae*, resembling in this respect a number of Gram-positive bacteria, including *Bacillus* (Fraser et al., 1995; Himmelreich et al., 1996). The highly conserved nature of the ribosomal protein genes has also found its use in establishing mollicute phylogeny (see the section Phylogeny and Taxonomy).

Organization of the mollicute rRNA genes generally follows the characteristic eubacterial order: 16S-23S-5S, functioning as an operon (Glaser et al., 1992). Some exceptions to the above classical rRNA gene organization have

been reported (Razin et al., 1998; Chambaud et al., 2001). Mollicute genomes carry only one or two rRNA gene sets (Table 5), whereas *Clostridium ramosum* and *Clostridium innocuum*, phylogenetically closest to mollicutes, have four and five sets of rRNA genes, respectively (Bove, 1993). Interestingly, phytoplasmas carry two rRNA gene sets, resembling in this respect their phylogenetic relatives, the acholeplasmas (see the section Phylogeny and Taxonomy).

Transfer Rnas The number of tRNA genes in mollicutes is kept to a minimum, with very few gene duplicates (Table 5). The set of 29 tRNA genes identified in the *M. pulmonis* genome represents the smallest set of tRNA genes among the sequenced genomes (Chambaud et al., 2001). Accordingly, the number of anticodons in the mycoplasmas is not much higher than in mitochondria and is close to the essential minimum for translation of all the amino acid codons by wobbling (Muto and Ushida, 2002).

Codon Usage Most mollicutes have genomes with a very low G+C content (Table 1), the outcome of an AT-biased directional mutation pressure (A-T pressure) during their evolution (Muto and Ushida, 2002). This has resulted in codon usage favoring synonymous codons with A and T, particularly in the wobble (3′) position. Codon bias in the AT-rich mycoplasmas is not limited to the third nucleotide position and is evident also in the first and second positions. Mycoplasmas have fewer GGN, CCN, GCN and CGN codons.

Another apparent outcome of codon reassignment under strong A-T pressure is the reassignment in most mollicutes of UGA from a stop codon to a tryptophan codon, a feature found in mitochondria (Osawa et al., 1992). As can be seen in Table 5, not all mollicutes share this property. The phylogenetically early acholeplasmas and phytoplasmas (see the section Phylogeny and Taxonomy) use the conventional UGG codon for tryptophan, carry tRNA^Trp (CCA), and retain UGA as a stop codon. As a consequence of using UGA as a tryptophan codon, it is difficult to express cloned mollicute genes in *E. coli*. Because *E. coli* regards UGA as a stop codon, translation of a mycoplasmal message in *E. coli* will stop where originally there should be tryptophan, so that mycoplasmal proteins expressed in *E. coli* may be truncated. One way to overcome this difficulty is by using an *E. coli* opal suppressor strain for expression (Renbaum et al., 1990; Smiley and Minion, 1993), though efficiency of expression falls considerably with increasing numbers of UGAs in the mycoplasmal message. Another way is to employ *Bacillus subtilis* that carries a tRNA that reads the UGA

termination codon as tryptophan (Kannan and Baseman, 2000).

In mitochondria, family codon boxes are each read by a single tRNA having an unmodified uridine at the first (or wobble) position of the anticodon. Similarly, *M. capricolum* and *M. mycoides* carry only a single isoacceptor tRNA for six of the eight family boxes (Andachi et al., 1989; Inagaki et al., 1995), and *M. pneumoniae* carries a single tRNA for each of the Ala, Leu, Pro and Val family boxes (Simoneau et al., 1993).

The elongation factor genes (*fus*, *efp*, *tsf* and *tuf*) were identified in the *M. genitalium* and *M. pneumoniae* genomes (Fraser et al., 1995; Himmelreich et al., 1996). Of these, the *tuf* gene, encoding the elongation factor EF-Tu, which mediates attachment of amino acyl-tRNAs to the ribosome, has attracted the most attention. It has been one of the first mollicute genes to be cloned, sequenced and expressed in *E. coli* (Yogev et al., 1990; Luneberg et al., 1991). Being a highly conserved housekeeping gene found in all mollicutes examined so far, it has been used as an effective genetic probe in taxonomy and phylogeny of mollicutes (see the section Phylogeny and Taxonomy). Of phylogenetic interest is the finding by Berg and Seemuller (1999a) that in the phytoplasma chromosome, the *fus* and *tuf* genes are linked in a transcriptional unit (the *str* operon) including also the ribosomal protein genes, *rps 12* and *rps 7*, resembling the chromosomal arrangement in *B. subtilis*. In members of the *Mycoplasma* genus, the *tuf* and *fus* genes are found in a different locus and are separately transcribed. This finding strengthens the notion that phytoplasmas are more closely related than the genus *Mycoplasma* is to the ancestral *Lactobacillus* group (see the section Phylogeny and Taxonomy).

Gene Transfer

The lack of a cell wall in mollicutes would be expected to facilitate the introduction of exogenous DNA into the cells. In fact, the exchange of chromosomal DNA during direct contact of mycoplasma cells (Barroso and Labarere, 1988) and conjugative transposition of transposon Tn*916* from *Streptococcus* (*Enterococcus*) *faecalis* to *M. hominis* (Roberts and Kenny, 1987) and to *M. gallisepticum* (Ruffin et al., 2000) by a spontaneous mating process, probably involves transient fusion of the cell membranes at the zone of contact. However, DNA transfer efficiency in these cases was rather low. Increased transformation and transfection efficiencies have been achieved either in the presence of polyethylene glycol (PEG) or by application of the electroporation procedure. Apparently, the method of choice depends on the mollicute species, and

on the particular DNA to be transferred (Dybvig and Voelker, 1996).

Genetic studies in mollicutes also have been hampered by the paucity of selectable markers (Dybvig and Voelker, 1996). Most studies on gene transfer in mollicutes have used as selectable markers the *tetM* tetracycline-resistance determinant found on Tn*916*, or the gentamycin-resistance determinant of Tn*4001*. The *tetM* gene is preferable as a marker because mollicutes in general are sensitive to tetracycline, whereas not all mollicutes are sensitive to gentamycin (Dybvig and Voelker, 1996). Thus, transposon Tn*4001* containing the *tetM* gene constructed by Dybvig et al. (2000) was found effective as a broad host-range vector, as indicated by the successful expression of a *lacZ* fusion gene in both *M. pulmonis* and *M. arthritidis*.

Gene function can be ascertained only by mutational analysis. The recent deluge of information on putatively identified mycoplasmal genes, based on sequence analysis (see the section Genome Sequencing and Gene Annotation), has emphasized the need for complementation of these predictions by mutational analysis. Having efficient transformation procedures at hand, major efforts have been directed at developing cloning and shuttle vectors for mollicutes (reviewed by Renaudin [2002]). Transfection experiments with the replicative form (RF) of the *S. citri* virus SpV1, carrying as an insert a segment of the gene coding for the P1 adhesin of *M. pneumoniae* (G-fragment) resulted in the expression of the fragment in the spiroplasma. In this case, the presence of seven UGA codons in the G-fragment insert did not interfere with expression, as *S. citri* can read UGA as tryptophan (Marais et al., 1993). Yet, the SpV1-RF/*S. citri* cloning system was deficient insofar as it suffered from the rapid loss of the cloned DNA insert. The loss of the insert was suggested to be the result of illegitimate and homologous recombination (Marais et al., 1993). An improved type of a cloning vector for *S. citri* was subsequently constructed by combining the *oriC* of *S. citri* with *tetM* and a colE1-derived *E. coli* replicon. The artificial recombinant plasmid, named "pBOT1," was able to replicate in *S. citri* and then integrate into the spiroplasmal chromosome, being stably maintained there (Renaudin, 2002). The pBOT1-derived, recombinant plasmids carrying heterologous DNA inserts were effective as shuttle vectors and, for example, restored fructose utilization in a mutant with a defective fructose operon (Gaurivaud et al., 2000a). Furthermore, similar *oriC* plasmids, developed for three mycoplasmas belonging to the *M. mycoides* cluster, showed that host specificity of these plasmids is not absolute, and closely related mollicutes exhibit a variable ability to replicate heterologous plasmids (Lartigue et al., 2003), indicating that *oriC*-based plasmids may serve as effective genetic vectors in mollicutes.

TRANSPOSITION The first successful transformation of *M. pulmonis* and *A. laidlawii* to antibiotic resistance was achieved by a plasmid, pAM120, harboring the streptococcal transposon Tn*916*, which contains the tetracycline-resistance determinant *tetM* (Dybvig and Cassell, 1987). The transposon was excised from the plasmid and integrated into the mycoplasmal chromosome at random sites, rendering the mycoplasmas resistant to tetracycline. Since then, a variety of mollicute species were shown to be transformed to tetracycline resistance by Tn*916* or Tn*1545*, or to gentamycin resistance, using the staphylococcal conjugative transposon Tn*4001*, which contains a gentamycin-resistance determinant (Voelker and Dybvig, 1996; Foissac et al., 1997a).

Transposon mutagenesis has already been applied rather effectively to generate mutants of *M. pneumoniae* and *M. genitalium* deficient in cytadherence (Krause et al., 1997) and of *S. citri* mutants deficient in motility and pathogenicity to plants (Foissac et al., 1997b; Jacob et al., 1997). Characterization of these mutants has already contributed significantly to the identification of genes and their protein products, associated with the mycoplasmal cytoskeleton and cytadherence (see the section Adhesion to Host Cells), spiroplasmal motility (see the section Morphology and Motility), and pathogenicity (see the section Pathogenicity). Doubtless, transposon mutagenesis will continue to be a most useful tool in mollicute genomic studies.

Though transposon mutagenesis is either random or directed to specific hot spots in the mycoplasmal genome, Dhandayuthapani et al. (1999) have introduced a technology enabling the targeting of mutagenesis to specific genes or operons. The technique is based on creation and delivery of disruption constructs acting by homologous recombination. The constructed plasmids contain the targeted gene and a gentamycin-resistance gene. Electroporation of the disruption plasmid, carrying part of the *mg218 M. genitalium* gene into wildtype hemagglutination-positive *M. genitalium*, permitted the isolation of hemagglutination-negative mutants, resulting apparently from homologous recombination at the *mg218* locus by single- or double-crossover events. In this way the role of the mg218 protein product in *M. genitalium* adherence could be demonstrated.

RESTRICTION AND MODIFICATION Restriction and modification of DNA allow a bacterium to distinguish between its own DNA and any foreign

DNA that lacks the characteristic host modification pattern. This difference renders an invading foreign DNA susceptible to attack by restriction enzymes that recognize the absence of methyl groups at the appropriate sites. Hence, these systems may pose a serious barrier to gene transfer. Restriction and modification systems in mollicutes have been recently reviewed by Sitaraman and Dybvig (2002). Generally, the restriction-modification (R-M) systems characterized in mollicutes in the past were defined as type II R-M systems, in which the endonuclease is separate from the methylase. More recently, *M. pulmonis* was found to possess a more complex type I R-M system, built of the S, M and R subunits. These subunits form a holoenzyme that performs both the endonuclease and DNA methylase activities (Dybvig et al., 1998).

CHROMOSOMAL REARRANGEMENTS The mollicute chromosome is a genetically dynamic structure undergoing frequent rearrangements, insertions, deletions, and inversions of genes or entire genomic segments. The mollicute genome may carry repetitive elements of various types, such as insertion sequence-like elements (ISLEs; see below), integrated viral or plasmid genomic segments, or repetitive elements of endogenous origin, constituting segments of mycoplasmal genes, as well as recombinases recently identified in mycoplasma genomes (Glew et al., 2002; Sitaraman et al., 2002; Flitman-Tene et al., 2003). These are the elements, which facilitate, through homologous recombination, chromosomal rearrangements as well as loss of genomic material by deletion of intervening sequences during recombination, a process presumably taking place during the reductive evolution of mollicutes (Rocha and Blanchard, 2002). The various types and mechanisms of chromosomal gene rearrangements in mycoplasmas have been extensively reviewed by Sitaraman and Dybvig (2002). Chromosomal rearrangements that play a crucial role in antigenic variation are discussed in the section Antigenic Variation.

INSERTION SEQUENCE-LIKE ELEMENTS Repetitive elements resembling the large insertion sequence (IS)*3* family of bacterial insertion sequences appear to be rather prevalent in mollicutes (Razin et al., 1998). However, of these, only IS*1138* was shown to actively transpose in *M. pulmonis* (Bhugra and Dybvig, 1993), whereas all the other elements that failed to show transposition within the mycoplasma chromosome are usually referred to as "IS-like elements" or ISLEs. The *M. pulmonis* IS*1138* is the only mycoplasmal ISLE shown to transpose in

the *M. pulmonis* chromosome at high frequency. The predicted amino acids encoded by the major IS*1138* ORF share significant similarity with the transposases of the IS*3* family, suggesting that IS*1138* has a potential for development into a cloning vector and mutagenesis vehicle in *M. pulmonis*. Notably, IS*1138* is species-specific to *M. pulmonis*, but it is ubiquitous among its strains (Bhugra and Dybvig, 1993).

Another species-specific ISLE is IS*1634*. It was recently identified in *M. mycoides* subsp. *mycoides* (small-colony type) and belongs to the IS*4* family. This pathogenic mycoplasma is distinguished by its exceptionally high number of insertion elements. Its genome was found to carry about 30 copies of IS*1634*, and about 20 copies of another, less specific IS*1296* element, comprising together about 7% of the total genomic DNA (Vilei et al., 1999). One may speculate that the numerous copies of these elements play a role in regulating the expression of the organism's genetic potential, including invasion ability into host tissues, characterizing this highly virulent mycoplasma (Vilei et al., 1999). A new type of an IS element, named "IS*1630*," was discovered in *M. fermentans* by Calcutt et al. (1999b). It belongs to the IS*30* family of transposons and has the striking ability to target and duplicate inverted repeats of variable length and sequence during transposition. In addition, the IS elements may influence gene expression, either by directly inactivating genes upon insertion or by providing promoters that increase the transcription of downstream genes. Insertion of ISLEs into mycoplasma genomes can be expected to mediate high-frequency genomic rearrangements. In fact, chromosomal rearrangement activity accompanied insertion of specific ISLEs into the chromosome of clinical isolates of *M. fermentans*. Insertion occurred at extremely high frequencies, as detected by genomic hybridization analysis of randomly subcloned colonies (Hu et al., 1998). More recently, *M. fermentans* was also found to carry a large (≈ 23-kb) genetic element that is present in four copies in the *M. fermentans* PG18 chromosome, accounting for approximately 8% of the genome (Calcutt et al., 2002). These novel elements, resemble conjugative, self-transmissible integrative elements (named "constins") and, surprisingly, are devoid of known integrases, transposases or recombinases, suggesting that a novel enzyme may be employed in excision and integration of these large elements. The skewed distribution and varied sites of chromosomal integration of the elements among *M. fermentans* isolates and in two other mycoplasma species, suggest that these elements play a role in promoting genomic and phenotypic variations in mycoplasmas.

Epidemiology and Control

Transmission

Acquisition of mollicutes can be established by direct contact between hosts or through secondary means, such as aerosols, fomites, food, water, insect vectors or other carriers, and by nosocomial acquisition (e.g., organ or tissue transplants; Tully, 1996). Many mycoplasmas inhabit the mucous membranes of the respiratory, urogenital, or gastrointestinal tracts of vertebrates, so that the mycoplasmas can be transmitted by direct oral-to-oral, genital-to-genital, or oral-to-genital contact. Thus, mycoplasmas found in the oropharynx may be found in some cases also in the genital tract and vice versa. Respiratory aerosols or fomites play apparently the major role in transmission of respiratory tract mycoplasmas, though neonates can be infected by *U. urealyticum* through in utero infection (Taylor-Robinson and Furr, 1998).

Food (and possibly water) obtained through feeding excursions plays a role in the acquisition of mollicutes by insects, where the organisms are present in plant sap or nectar, or where such materials are contaminated with other insect-derived excretions. In some predatory insects, the mycoplasmas are acquired directly by eating other infected hosts. Insect vectors are responsible for transmission of spiroplasma and phytoplasmas from plant to plant. These transmissions usually involve a biological cycle within the insect, involving multiplication of the mollicutes in various insect tissues (adipose tissue, salivary gland, etc.) followed by reinoculation into susceptible plants (Tully and Whitcomb, 1992).

Secondary transmission of mycoplasmas can also occur through healthy carriers or through convalescent carriers recovering from acute infections, as apparently occurs in some *M. pneumoniae* respiratory infections in humans and probably in acute and chronic respiratory mycoplasmal diseases in domestic animals. Infected carriers also play an important role in the transmission of *Spiroplasma melliferum* and *S. apis* infections from foraging bees to their hives. Mollicute transmission after plant grafting of infected tissue to healthy rootstock (Tully and Whitcomb, 1992) or through human organ or tissue transplantation have also been described (Tully, 1996). Semen contaminated with mycoplasma is an important factor in dissemination of sexually transmitted diseases in a variety of hosts and should be considered particularly in artificial insemination programs in humans and bovines (Kanakas et al., 1999).

The upper respiratory and genital tracts of human newborns are first colonized with mycoplasmas acquired during passage through the female birth canal. There is now substantial evidence that mycoplasma colonization can occur even earlier in utero by organisms introduced during fertilization or during early pregnancy. Generally, neonatal colonization with both human oral and genital mycoplasmas does not persist and the number and type of mycoplasmas decrease with age. Subsequent reacquisition of the human urogenital mycoplasma flora appears to be directly related to sexual contacts (Taylor-Robinson and Furr, 1998; Blanchard and Bebear, 2002). In vertebrates, acquisition from the maternal genital tract, and in utero transmission (including egg transmission in birds), apparently represents the initial mechanism of infection. Transovarian transmission of spiroplasmas has been well established in *Drosophila* species (Williamson and Poulson, 1989).

Host factors also play an important and complex role in determining whether an acquired mycoplasma behaves as a pathogen or is avirulent. Endogenous mycoplasmas may become pathogenic when the human host immune system has been compromised or when surgical intervention or trauma allows mycoplasma commensals to enter the circulation or other host tissues. Common commensals, such as *M. hominis*, *M. salivarium* and *U. urealyticum* have been shown to induce septicemia and overt mycoplasma invasion of various host tissues and organs, with localization in joints being especially prominent (Blanchard and Bebear, 2002).

Because *M. pneumoniae* infections are so common in humans, epidemiological data on these infections are abundant. One of the most puzzling features of *M. pneumoniae* pneumonia is the age distribution of patients. In a survey conducted between 1964 and 1975 of more than 100,000 individuals in the Seattle area, the age-specific attack rate was highest among 5- to 9-year-old children (Foy et al., 1979). Rates of *M. pneumoniae* pneumonia in the youngest age group, 0–4 years old, were about one-half those in school-age children, but considerably higher than those in adults. *Mycoplasma pneumoniae* pneumonia was rarely observed in infants younger than 6 months, suggesting maternally conferred immunity (Fig. 19). *Mycoplasma pneumoniae* accounts for 8–15% of all pneumonias in young school-age children. In older children and in young adults, the organism is responsible for approximately 15–50% of all pneumonias. Infection with *M. pneumoniae* is worldwide and endemic; it occurs all year round but shows a predilection for the colder months, apparently because of the greater opportunity for transmission by droplet infection. *Mycoplasma pneumoniae* appears to require close personal contact to spread; successful spreading usually occurs in
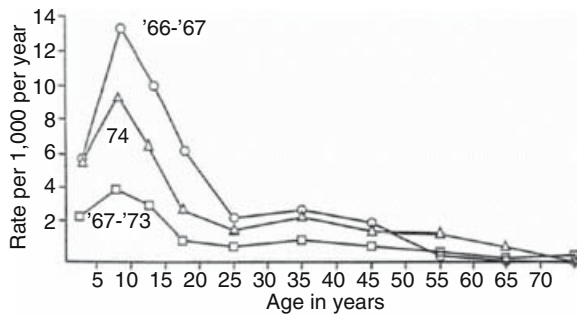
Fig. 19. Incidence of *M. pneumoniae* pneumonia in Seattle by age, for two epidemics (1966–67 and 1974) and the endemic period (1967–73). From Foy et al. (1979).

families, schools and institutions. The incubation period is relatively long, ranging from 2–3 weeks (Talkington et al., 2001; Jacobs, 2002).

Ureaplasma urealyticum is spread primarily through sexual contact. Colonization has been linked to the frequency of sexual intercourse and the number of sexual partners. Women may be asymptomatic reservoirs of infection (Taylor-Robinson and Furr, 1998).

Prevention

The frequent failure of antibiotics and other therapeutic approaches to eradicate mycoplasmas and abort the infectious disease process has led to the conclusion that development of effective vaccines is the most promising approach to control mycoplasma infections in humans and animals. The many efforts to develop effective and safe mycoplasmal vaccines have been only partially successful. The overall success obtained with formalin-inactivated *M. pneumoniae* vaccines in humans has been extremely disappointing, although fully inactivated vaccines and live attenuated vaccines have had some success in control of some mycoplasma diseases in animals. No acceptable live vaccine has been developed for human use (reviewed in Ellison et al. [1992]). Prior natural infection appears to provide the most effective resistance; however, evidence shows that *M. pneumoniae* infections recur at intervals of several years (Lind et al., 1997). These observations suggest that immunity to a single natural infection is relatively short-term, particularly in children, and it may be unrealistic to expect more, or even as much, from artificially induced immunity.

Attenuation of mycoplasma strains tends to reduce virulence and immunogenicity. In most cases, attenuated viable vaccines do not reach the level of protective efficiency required from a commercial vaccine. Killed *M. pneumoniae* vaccines administered intranasally to hamsters are relatively inefficient unless boosted by parenteral inoculation of vaccine (Ellison et al., 1992). Intranasal immunization may be ineffective because the antigenic mass is not retained for a sufficient period in the lungs. On the other hand, parenteral killed vaccines, particularly if combined with adjuvant, do produce adequate protection in terms of reducing pneumonia but have only a minimal effect on the number of organisms growing in the lungs. A similar protective effect can be achieved briefly by inoculation with hyperimmune serum. In summary, a single dose of vaccine in a form suitable for clinical use is unlikely to produce lasting immunity to *M. pneumoniae* infection. Stimulation of systemic antibodies may prevent the clinical manifestations of pneumonia, but additional local stimulation with live or killed organisms may be necessary to evoke resistance to colonization. The advantages of live vaccines are well established, but they frequently suffer from some deficiencies. Thus, the live *M. gallisepticum* F-strain provides good protection but may be pathogenic to young chicks. Vaccines based on temperature-sensitive mutants of *M. gallisepticum* are safer than the F-strain but do not afford the same level of protection. Consequently, Papazisi et al. (2002b) proposed a new approach, based on employing a live, attenuated *M. gallisepticum* strain R_{high} that had lost the *gapA* and *crmA* adhesin genes. Insertion of the adhesin *gapA* gene into the chromosome of the attenuated strain resulted in the expression of a high level of Gap A on the mycoplasma cell surface. Vaccination of chicks with this genetically modified strain produced a high level of antibodies to Gap A, protecting the chicks against infection with the virulent R_{low} *M. gallisepticum*.

An approach worth pursuing is the preparation of vaccines made of antigenic components specifically related to the mycoplasma-host cell interaction, such as mycoplasmal adhesins. Guinea pigs preimmunized with purified P1 adhesin protein and subsequently infected with *M. pneumoniae* showed increased levels of *M. pneumoniae*-specific IgG, IgA and adherence-inhibiting antibodies. However, these animals developed severe lung lesions on challenge, suggesting that it may well be harmful to vaccinate the host with the P1 protein, inasmuch as it may sensitize the host to the extent that subsequent infection will potentiate host response and lead to a more severe disease (Jacobs et al., 1988; Razin and Jacobs, 1992). Another mycoplasmal protein vaccine was developed more recently against *M. hyopneumoniae* (Chen et al., 2001). This vaccine was composed of a recombinant chimera of the nontoxic moiety of *Pseudomonas* exotoxin with a fragment of a *M. hyopneumo-*

niae cytadhesin overexpressed in *E. coli*. The serological response of pigs to this chimeric protein was promising, but more work is needed to show the effectivity of this vaccine under field conditions.

DNA Vaccines Genetic immunization (DNA vaccines) is a novel promising approach to vaccine production that has many of the advantages of live and attenuated vaccines but with no risk of infection. Confounding factors, such as adjuvant, hapten carriers, or denaturing agents are not needed. Vaccine production involves introducing DNA encoding a pathogen protein into host cells, using an expression library of the pathogen. The first step in the process is the successful transfection of cells that take up the plasmid. The cDNA, with a strong mammalian promoter in the construct, directs the synthesis of mRNA, leading to the synthesis of the immunogen protein. If the transfected cell is a professional antigen-presenting cell (APC, e.g., a dendritic cell) that expresses class I and II MHC proteins as well as co-stimulatory molecules, the process of antibody production can proceed (Lai and Bennett, 1998). The DNA vaccine induces effectively antibodies as well as cytotoxic T lymphocytes. Thus both humoral and cell-mediated responses are induced—a distinct advantage. Barry et al. (1995) prepared an *M. pulmonis* library by fusing digested mycoplasmal DNA onto the last exon of the gene encoding human growth hormone (hGH), using a cytomegalovirus vector, so that hGH-*M. pulmonis* antigens might be secreted. Theoretically, a library expressing the entire genome of a pathogen could be used as a vaccine. Barry et al. (1995) found that about 1 ng DNA was required to produce an immune response by genetic immunization into the skin. Mice immunized with *M. pulmonis* libraries and challenged had no culturable mycoplasmas and no lung lesions.

Lai et al. (1994) constructed an *M. pulmonis* genomic library cloned in λgt 11 transfected into *E. coli*. Clones of *E. coli* expressing a fusion protein reactive with anti-*M. pulmonis* antibodies were transferred orally or intravenously into mice. Expression of fusion proteins in the mice was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) into the drinking water, resulting in local and systemic antibody production and effective protection of the animals against large numbers of virulent *M. pulmonis*. DNA vaccines also could be used to treat animals already infected with *M. pulmonis*. When infected mice were immunized with the DNA vaccine one week after infection, the numbers of organisms recovered from tracheolung lavage fluids gradually decreased and were totally eliminated by four months (Lai et al., 1997).

The epitope important as an immunogen may be selected by isolating the dominant antigen, preparing a monoclonal antibody to it, and using this antibody to select the *E. coli* clone producing the specific epitope-bearing fusion protein. Thus, the DNA vaccine is controllable. Such a specific DNA vaccine was developed for *M. hyopneumoniae*. Following screening of the mycoplasmal clone bank with hyperimmune pig serum, Fagan et al. (1996) identified a clone with a sequence homology to the R2 subunit of ribonucleotide reductase. This clone was expressed in *E. coli* as an 11-kDa protein fused to β-galactosidase. Assessment of the vaccine potential of the fusion protein in pig trials showed the vaccinated pigs to be significantly protected against challenge with a virulent *M. hyopneumoniae*. An attenuated *Salmonella typhimurium aroA* strain used as a vector for delivery of the above immunogenic *M. hyopneumoniae* fusion protein did protect mice against experimental infection with *M. hyopneumoniae* and is thus a potentially cheap and easily adminstrable oral vaccine for swine (Fagan et al., 1997). Efforts to develop an effective DNA vaccine against *M. hyopneumoniae* are still under way, suggesting the failure of the above-described DNA vaccines to provide satisfactory protection of swine in field trials. A recent trial is that reported by Chen et al. (2003). They constructed a plasmid vector carrying the P42 heat shock protein gene of *M. hyopneumoniae* driven by the potent cytomegalovirus promoter. Injection of the plasmid into mice induced both humoral and cellular responses. The antiserum from the immunized mice inhibited growth of *M. hyopneumoniae*. One can only hope that this vaccine will offer better protection of swine than the previous DNA vaccines.

A novel genetic strategy to control phytoplasma diseases has been based on expression in plants of antibodies (plantibodies) that are able to interfere with multiplication of the pathogen. Le Gall et al. (1998) engineered a gene of a single-chain variable fragment antibody to a major membrane protein of the stolbur phytoplasma. They cloned it in a plasmid of *Agrobacterium tumefaciens* and introduced it into tobacco plants. The transformed plant carried the transgene. When grafted on a stolbur-infected tobacco root stock, the transgenic tobacco shoots grew free of symptoms, while normal tobacco shoots showed severe stolbur symptoms.

Because the great potential of DNA vaccines is not in doubt, rapid development of this new generation of vaccines can be expected. Whether the high hopes for these vaccines are justified will have to await the results of large-scale field trials.

TREATMENT The mycoplasmas are sensitive to most broad-spectrum antibiotics (such as the tetracyclines and macrolides, as well as the newer quinolones) but are resistant to antibiotics that specifically inhibit bacterial cell wall synthesis. Susceptibility profiles of human mycoplasmas to a variety of the classical as well as the newer antibiotics can be found in Hannan (2000) and Bebear and Bebear (2002).

Tetracyclines are widely used in treatment of genital tract infections, but macrolides are also widely used for respiratory tract infections. Erythromycin, the newer macrolides, ketolides, and newer quinolones (when compared with tetracyclines) have equal or sometimes greater activity against mycoplasmas. Most of the effective antibiotics only inhibit mycoplasma multiplication but do not kill them. The ketolides and some quinolones also have some mycoplasmacidal activity (Taylor-Robinson and Bebear, 1997; Waites et al., 2003).

Mycoplasmas may develop resistance, either by gene mutation, by acquisition of a resistance gene, or by active efflux of the antimycoplasmal agent (Bebear and Bebear, 2002; Pereyre et al., 2002; Raherison et al., 2002; Reinhardt et al., 2002). Thus, integration of the *tetM* gene into the *U. urealyticum* or the *M. hominis* chromosome renders these mycoplasmas resistant to tetracycline. The resistance factor could be transferred from other tetracycline-resistant bacteria by a process resembling conjugation (Roberts and Kenny, 1987).

Mycoplasmas may be difficult to eradicate from human or animal hosts or from contaminated cell cultures by antibiotic treatment, because specific mycoplasma are either resistant to the antibiotic, lack mycoplasmacidal activity, or are in an intracellular location (see the section Intracellular Location). As expected, mycoplasma eradication is particularly difficult in immunosuppressed or immunodeficient patients.

Pathogenicity

Most mollicutes live as commensals, and in many arthropods they may even be considered as symbionts. In the case of pathogenic mycoplasmas, infections are rarely of the fulminant type but rather follow a chronic course. Mycoplasmas are arguably close to "ideal parasites," usually living in harmony with their host. Extensive descriptions of the diseases caused by mollicutes in humans, animals, plants and insects can be found in *The Mycoplasmas*, volumes IV (Razin and Barile, 1985) and V (Whitcomb and Tully, 1989), in Maniloff et al. (1992) and in Razin and Herrmann (2002). A variety of diseases of unknown etiology were also linked to mycoplas-

mas (Baseman and Tully, 1997). These include the possible role of mycoplasmas as cofactors to AIDS pathogenesis, Gulf War syndrome, and other diseases of unexplained etiology, such as chronic fatigue syndrome, Crohn's disease, and various arthritides (Lo et al., 2000; Endresen, 2003). Although the association of mycoplasmas with many of these diseases remains doubtful, the possible role of mycoplasmas in AIDS activation has attracted the most attention during the last decade. The substantial number of studies carried out on the AIDS-associated mycoplasmas, *M. fermentans* and *M. penetrans*, have advanced considerably our knowledge of mycoplasma cell biology and host immune modulation by mycoplasmas, as evidenced in many sections of this chapter. However, thus far the proposed role of mycoplasmas in AIDS activation remains controversial and doubtful (Brenner et al., 1996; Baseman and Tully, 1997; Taylor-Robinson and Furr, 1998).

Mycoplasmas were suspected long ago as causative agents of rheumatoid arthritis (RA), but solid evidence to support this was lacking. The recent emphasis on the immunomodulatory effects by mycoplasmas (see the section Immune System Modulation) and more so, detection of mycoplasmal DNA, particularly that of *M. fermentans*, in synovia of RA patients (Schaeverbeke et al., 1999) as well as the prevalence of antibodies to *M. fermentans* in the patients (Horowitz et al., 2001) suggest that in some RA patients *M. fermentans* may play a role in initiating or perpetuating synovitis (Blanchard and Bebear, 2002).

Disease Manifestations

The effects of *M. pneumoniae* on humans include subclinical infection, upper respiratory disease, and bronchopneumonia. Most human infections do not progress to a clinically evident pneumonia. When pneumonia occurs, the onset generally is gradual and the clinical picture is one of mild to moderately severe illness, with early complaints referable to the lower respiratory passages. Radiography frequently reveals evidence of pneumonia before physical signs are apparent. Involvement is usually limited to one of the lower lobes of the lungs, and the pneumonia is interstitial or bronchopneumonic. The course of disease varies; remittent fever, cough and headache persist for several weeks. One of the most consistent clinical features is a long convalescence, which may extend 4–6 weeks. Few fatal cases have been reported. Several complications following *M. pneumoniae* infections have been noted, including hemolytic anemia, polyradiculitis, encephalitis, aseptic meningitis, and central nervous system illness such as the Guillain-Barré

syndrome. In addition, pericarditis and pancreatitis have been observed. Although these sequelae have been commonly related to the suspected immunopathology of *M. pneumoniae* disease, *M. pneumoniae* has been increasingly demonstrated in the affected organs by PCR, or in some cases even by culture (Narita et al., 1995; Bencina et al., 2000).

Growing evidence suggests that *Ureaplasma urealyticum* causes nongonococcal urethritis in men that are free of *Chlamydia trachomatis*, an established agent of nongonococcal urethritis. The wide occurrence of *U. urealyticum* in sexually active, symptom-free adults hampers research in this field. Evidence is based primarily on the production of nongonococcal urethritis symptoms in ureaplasma-free and chlamydia-free volunteers by intraurethral inoculation of *U. urealyticum*. Ureaplasmas have also been associated with chorioamnionitis, habitual spontaneous abortion, and low-weight infants (Gerber et al., 2003). *Mycoplasma hominis*, a common inhabitant of the vagina of healthy women, becomes pathogenic once it invades the internal genital organs, where it may cause pelvic inflammatory diseases such as tubo-ovarian abscess or salpingitis (Taylor-Robinson and Furr, 1998).

Mycoplasma genitalium, isolated in 1981 from the urethral discharge of two homosexual men, may account for the tetracycline-responsive, nongonococcal urethritis cases in which chlamydias and ureaplasmas could not be isolated (about 20% of all cases). However, *M. genitalium* is so fastidious that very few clinical isolates have so far been made on the best mycoplasma media available. Only the recent application of specific PCR amplification of the organism's DNA in clinical specimens has provided experimental proof for the relative prevalence of *M. genitalium* in the human urogenital tract and its apparent role in chronic male urethritis (Taylor-Robinson and Furr, 1998; Totten et al., 2001) and possibly also in acute endometritis and cervicitis in women (Cohen et al., 2002; Manhart et al., 2003).

Many *Mycoplasma* species are established pathogens of farm animals, causing contagious pleuropneumonia, mastitis, and conjunctivitis in cattle, goats and sheep, and chronic respiratory disease and arthritis in swine, chicken and laboratory animals (Frey, 2002).

Molecular Basis of Mycoplasma Pathogenicity

OXIDATIVE DAMAGE AND MEMBRANE FUSION Potent toxins have not been associated with mycoplasmas. The mildly toxic byproducts of mycoplasma metabolism (such as hydrogen per-
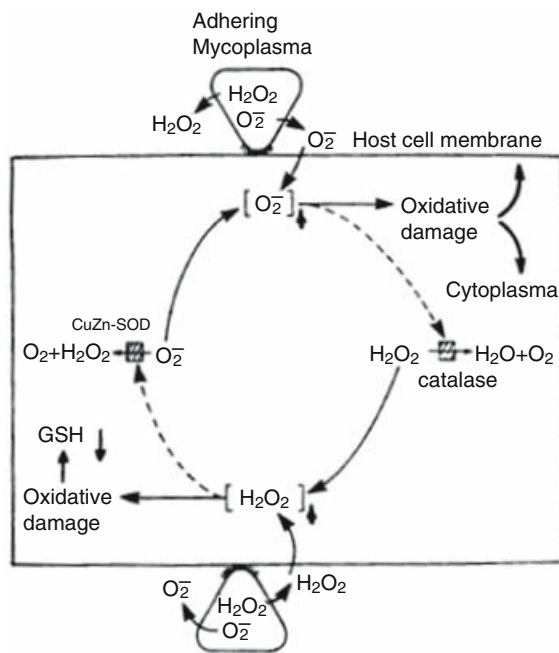
Fig. 20. Schematic presentation of the proposed mechanism for oxidative tissue damage by mycoplasmas. GSH, glutathione and SOD, superoxide dismutase. From Kahane (1984).

oxide and superoxide radicals) have been accused of causing oxidative damage to host cell membranes. Extensive studies by Almagor et al. (1986) led to the formulation of the following pathological events of *M. pneumoniae* infection (Fig. 20). The H_2O_2 and superoxide radicals (O_2^-) continuously generated by the adhering mycoplasmas penetrate into the host cell. As a result of O_2^- accumulation, gradual irreversible inhibition of host cell catalase is induced, thereby causing intracellular H_2O_2 accumulation. The latter, in turn, may cause product inhibition of the host cell superoxide dismutase. The process is self-perpetuating and results in increased levels of O_2^- and H_2O_2, which induce progressive oxidative damage to vital cell constituents. Thus, malonyldialdehyde, a known oxidative product of membrane lipids, was shown to accumulate in cells infected by *M. pneumoniae*. Supportive of the role of hydrogen peroxide in mycoplasma pathogenicity are the recent findings of Vilei and Frey (2001) showing that the higher virulence of the African strains of *M. mycoides* subsp. *mycoides* SC may be associated with their ability to transport and metabolize glycerol. Peroxide production in glycerol-containing growth media was high for the African strains but very low for the much less virulent European strains that lack the genes for glycerol transport. A question most frequently asked is how the mycoplasmas, lacking antioxidants like catalase and superoxide

dismutase, protect themselves against oxidative stress. The finding by Dhandayuthapani et al. (2001) of methionine sulfoxide reductase in *M. pneumoniae* and *M. genitalium*, an antioxidant enzyme that catalyzes the reduction of methionine sulfoxide residues in proteins to methionine, may play an important role in protecting mycoplasma protein structure from oxidative damage.

The lack of a cell wall in mycoplasmas is expected to facilitate direct contact of the mycoplasma membrane with that of its eukaryotic host, creating a condition which, in principle, could lead to fusion of the two membranes, enabling transfer or exchange of membrane components and injection of mycoplasmal cell constituents into the host cell (Razin et al., 1998; Rottem, 2002c). Unique *M. fermentans* glyceroglycolipids containing phosphocholine (Rottem, 2002a) exhibit fusogenic properties, in addition to serving as important surface immunogens inducing cytokine secretion. The possibility that the synthesis of the choline-containing lipids by *M. fermentans* may cause choline depletion in the host has been suggested by Ben-Menachem et al. (2001). Choline is an essential component of eukaryotic cell membranes. *Mycoplasma fermentans* was found to deplete the choline component of an astrocyte cell culture leading to apoptosis of the cells, an effect that could be prevented by the addition of free choline to the growth medium. Whether choline deficiency can occur in patients infected by *M. fermentans* is obviously a moot point.

INTRACELLULAR LOCATION The uptake of human and animal mycoplasmas by polymorphonuclear leukocytes and macrophages was shown (Marshall et al., 1995), and intracellular location of spiroplasmas and phytoplasma within salivary gland cells of insects is an integral part of their life cycle. However, the question of whether animal mycoplasmas can enter epithelial cells has not been easy to resolve, and for a long time, this question was answered in the negative. The stimulus to re-examine this issue came from the studies by Lo (1992) showing the intracellular location of *M. fermentans* (strain incognitus) in a variety of nonphagocytic cells in AIDS patients. This finding was strengthened by the discovery of a new human mycoplasma capable of entering a variety of human cells in vivo and in vitro, named accordingly *M. penetrans* (Lo et al., 1993; Fig. 21).
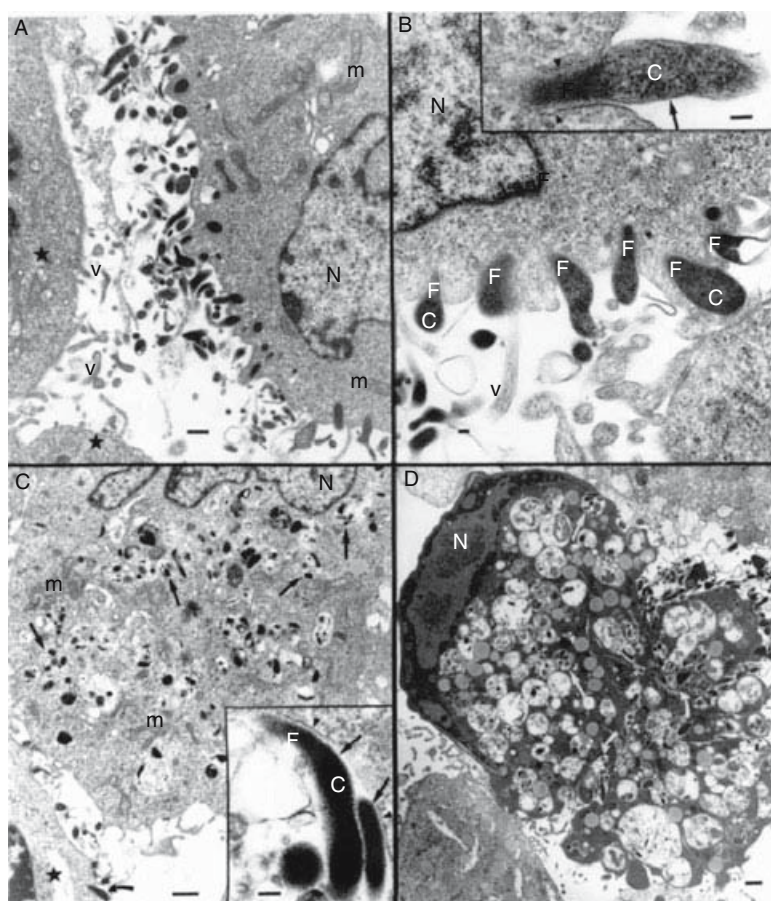
Fig. 21. Adhesion and invasion of mammalian cells by *Mycoplasma penetrans*. Many organisms line along the cell surface with their tip-like structure of densely packed fine granules compartment (F) inserted into the eukaryotic cell. The body compartment of loosely packed coarse granules (C) remains outside. Inset shows the active invasion process at higher power. Bars represent 100nm. From Lo et al. (1993).

The mechanism of cell entry by mycoplasmas is still unclear. Even though mycoplasmas, such as *M. penetrans* and *M. genitalium* appear to enter the cells through their specialized tip structure (Lo et al., 1993; Jensen et al., 1994), other mycoplasmas shown to internalize (e.g., *M. fermentans* and *M. hominis*) have no tip structures (Taylor-Robinson et al., 1991). Following contact of *M. genitalium* with human lung fibroblasts, the plasma membrane of the cells appeared to be forced inward to form a cup or a depression. The membrane pockets resembled clatherin-coated pits, suggesting that the mycoplasma might adhere to and enter the cells by a site-directed, receptor-mediated event, resembling cell entry by chlamydia (Mernaugh et al., 1993). Host cell entry appears to depend on the assembly of actin microfilaments and organized microtubules, as cytochalasin D prevented invasion by *M. penetrans* (Rottem, 2002c). Apparently, *M. penetrans* stimulates host cell phospholipases to cleave membrane phospholipids, thereby initiating a signal transmission cascade. This cascade triggers cytoskeletal rearrangements, allowing the internalization of *M. penetrans*. The presence in the host cell cytoplasm of mycoplasmas, some of which may not even be enclosed within a vacuole, may expose the cytoplasm and the nucleus to mycoplasmal hydrolytic enzymes, such as proteases, nucleases and phospholipases (Rottem, 2002c).

Whether the mycoplasmas replicate intracellularly remains to be resolved. The study by Dallo and Baseman (2000) indicated that intracellular *M. pneumoniae* and *M. penetrans* infectivity in human cells could be detected for over 6 months, while *M. genitalium* survival in the cells was detected by PCR for long periods. Moreover, specific inhibition of eukaryotic nuclear DNA synthesis by aphidicolin enabled the selective detection of mycoplasmal DNA synthesis, supporting their intracellular replication (Dallo and Baseman, 2000).

Notably, intracellular location, if even for a short period, may protect the mycoplasmas against the effects of the host immune system and antibiotics and, to some extent, account for the difficulty of eradicating mycoplasmas from infected cell cultures. Thus, intracellular residence, which sequesters mycoplasmas, promotes the establishment of latent or chronic infection states, and circumvents mycoplasmacidal immune mechanisms and selective drug therapies (Bebear and Bebear, 2002). Different aspects of the interaction of mycoplasmas with host cells, including fusion of the parasite with the host cell membrane, invasion into host cells, and the implications of these processes on mycoplasma pathogenicity, have been recently reviewed in depth by Rottem (2003).

CLASTOGENIC AND ONCOGENIC EFFECTS The potent mycoplasmal nucleases, combined with superoxide radicals, may be responsible for clastogenic effects. In fact, reports on chromosomal aberrations, altered morphology, and cell transformation in cell cultures infected by mycoplasmas have appeared rather sporadically since the early 1960s. Interest in this subject has been rekindled following the claims that *M. penetrans* may be a cofactor in the induction of HIV-associated Kaposi's sarcoma. Results of experiments with cultured mouse embryo cells reported by Tsai et al. (1995) and by Feng et al. (1999b) appear to support an oncogenic potential of the AIDS-associated mycoplasmas *M. fermentans* and *M. penetrans*. However, instead of acute transformation, a multistage process in promotion and progression of malignant cell transformation with long latency was noted. Only after 18 passages (one week per passage) of persistent infection with mycoplasmas, an irreversible form of transformation, that included the ability to form tumors in mice, was achieved. Of note, however, is that the tumors had developed very slowly and not in all mice. The irreversible phase of transformation coincided with permanent karyotypic alterations. Once induced, chromosomal alterations continued to accumulate both in cultured cells and in animals without the continued presence of the transforming mycoplasmas. Hence, the mycoplasma-mediated multistage oncogenesis exhibited many characteristics found in the development of human cancer (Lo, 2002). Nevertheless, the real biological significance of these findings warrants further investigation.

Another related issue concerns the induction of apoptosis by mycoplasma infection (reviewed by Lo [2002]). Endogenous nucleases are generally considered to catalyze DNA fragmentation, a common biochemical hallmark of apoptosis. In light of the relatively large amounts of nucleases expressed by mycoplasmas (Bendjennat et al., 1999), Paddenberg et al. (1998) tested the effects of *M. hyorhinis* infection of NIH 3T3 cells, checking for parameters characteristic of apoptosis. The infected cells exhibited intranucleosomal DNA degradation into multimers of 200 bp, forming a ladder in agarose gels. Nuclease activities were detected in cell homogenates and culture supernatants. Their mycoplasmal origin was indicated by inhibition of their expression following chloramphenicol treatment of the infected cells. Furthermore, enriched mycoplasmal nucleases, purified from culture supernatants of *M. hyorhinis* and *M. penetrans* could reproduce the apoptotic effects of mycoplasmal infection (Paddenberg et al., 1998; Bendjennat et al., 1999). Apoptotic effects were also observed in lymphocyte and epithelial tumor cell lines

infected by mycoplasmas, pointing to the possibility that the numerous cases of "spontaneous apoptosis" reported in cell cultures are, in fact, the results of an undetected mycoplasma infection (Paddenberg et al., 1998; Stolzenberg et al., 2000). The finding that *U. urealyticum* induces apoptosis in human lung epithelial cells and macrophages may support the proposed role of the ureaplasmas in chronic lung disease of premature infants (Li et al., 2002). Some insight into the mycoplasmal cell components that induce apoptosis has been presented by Into et al. (2002) showing that mycoplasmal membrane lipoproteins induce necrotic and apoptotic cell death in monocytes-macrophages and lymphocytes. Apoptosis was induced by caspases (cysteine proteases) through TLR2 (Toll-like receptor 2)-mediated signaling.

PATHOGENCITY ASSOCIATED GENES A promising genetic approach, based on transposon Tn*4001* mutagenesis, has been applied to study pathogenicity factors of *S. citri* (Foissac et al., 1997b; Gaurivaud et al., 2000a, b). One of the Tn*4001* mutants did not multiply in the leafhopper vector and therefore could not be transmitted to the plant. Another mutant multiplied well in the plant but did not induce symptoms. In this non-pathogenic mutant, Tn*4001* was found inserted in the spiroplasmal PTS fructose operon, abolishing the ability of the organism to utilize fructose, probably affecting in this way its pathogenicity. The most likely explanation is that utilization of fructose in the plant sieve tubes by the pathogenic spiroplasmas may interfere with the normal physiology of the plant causing chlorosis, stunting and wilting (Bai and Hogenhout, 2002). Of interest would be whether pathogenicity of the mutant can be restored by complementation with the wildtype genes, like the successful restoration of motility in a Tn*4001* *S. citri* mutant by complementation with the wildtype genes (Jacob et al., 1997; see the section Motility and Cytoskeletal Elements). In continuation of this line of research, Jagoueis-Eveillard et al. (2001) embarked on the study of plant genes that are deregulated following infection by mollicutes. These rather preliminary studies revealed that cDNAs of periwinkle plants are differently expressed following infection with *S. citri* or by phytoplasmas. Clearly, much more work has to be carried out to identify the metabolic pathways modified during plant-mollicute interactions, but the genetic approach appears to be very promising.

The lysogenic bacteriophage MAV1 infecting *M. arthritidis* (Voelker and Dybvig, 1999; Washburn and Cole, 2002) appears to play an important role in the pathogenicity of this murine mycoplasma. All virulent *M. arthritidis* strains carry MAV1 DNA integrated at various sites of the mycoplasmal chromosome, whereas avirulent strains lack MAV1. Furthermore, the arthritogenic potential of a low-virulence *M. arthritidis* strain could be considerably enhanced by lysogenization with MAV1 (Voelker et al., 1995). The mechanism by which MAV1 enhances virulence is not known. The fact that several MAV1-negative strains are not completely avirulent suggests that MAV1 integration may activate other virulence factors, such as the *M. arthritidis* superantigen, (known as MAM; see the section Immune System Modulation), or increase the expression of *M. arthritidis* cytadhesins. The recent identification of an MAV1 gene that apparently encodes a lipoprotein may provide some direction to future research, by assuming that the phage-derived lipoprotein is incorporated into the cell membrane of the avirulent *M. arthritidis* and induces in some unknown way the conversion of the avirulent to the virulent phenotype (Voelker and Dybvig, 1999; Tu et al., 2002).

Adhesion to Host Cells

Most human and animal mycoplasmas adhere tenaciously to the epithelial linings of the respiratory or urogenital tract, rarely invading tissues. Hence, they may be considered as surface parasites. Adhesion of mollicutes to host cells is a prerequisite for colonization by the parasite and for infection. The loss of adhesion capacity by mutation results in loss of infectivity, and reversion to the cytadhering phenotype is accompanied by regaining infectivity and virulence. The best-defined mycoplasmal adhesins (membrane components responsible for adhesion) are those of *M. pneumoniae* and *M. genitalium*. Detailed information on the genes and molecular properties of the encoded adhesin proteins of these mycoplasmas (P1, MgPa and P30) can be found in several reviews and articles (Razin and Jacobs, 1992; Razin et al., 1998; Krause and Balish, 2001; Balish and Krause, 2002; Musatovova et al., 2003).

Although the above-mentioned adhesins fulfill the major role in cytadhesion of *M. pneumoniae* and *M. genitalium*, the process appears to be multifactorial, involving in addition to the major adhesins, a number of accessory membrane proteins. These accessory proteins act in concert with cytoskeletal elements to facilitate the lateral movement and concentration of the adhesin molecules at the attachment tip organelle and provide the scaffolding for the assembly of this organelle (see the section Motility and Cytoskeletal Elements). Much of the recent effort, forming part of the *M. pneumoniae* genome project, has been directed to the molecular definition of

the attachment organelle components, forming part of the mycoplasmal cytoskeleton ("Triton shell"). Some of the genes and the encoded proteins of the *M. pneumoniae* Triton shell (30-kDa, 40-kDa, 90-kDA, P65, P200, HMW1, HMW2 and HMW3) have been identified and characterized. Interestingly, they are proline rich and exhibit repeat sequences and other motifs characteristic of eukaryotic cytoskeletal proteins. Thus, the C-terminal domain of P30 shows substantial sequence homology with the C-terminus of P1 and exhibits immunological crossreactivity with fibrinogen, keratin and myosin—a finding which may be associated with the autoimmune sequelae following *M. pneumoniae* infections (Romero-Arroyo et al., 1999).

The HMW1 protein seems to be responsible for targeting (trafficking) the P1 adhesin to the attachment organelle (Balish et al., 2001), whereas the 40- and 90-kDa proteins anchor P1 to the membrane of the organelle in a way that allows mutual and intimate interactions of P1 with cytoskeletal-forming or -associated proteins. Loss of the 40- and 90-kDa proteins by mutation results in the loss of the attachment organelle, cells becoming spherical and nonadherent (Layh-Schmitt and Harkenthal, 1999). The genes for HMW1 and HMW3 are part of the *hmv1* locus, along with the gene for P30. The *hmv2* gene for HMW2 lies about 160kb from the *hmw1* locus. Yet, transposon insertions into *hmw2* resulted in the simultaneous loss of HMW1, HMW3 and P65 as well as of several other cytadherence-associated proteins, probably due to accelerated endogenous proteolysis (Jordan et al., 2001; Krause and Balish, 2001). Thus, HMW2 apparently functions as a cytoskeletal organizer and is required for the stable maintenance of the above proteins (Balish et al., 2003). The complex interrelationship between the components of the attachment organelle underscores the need for the proper ordered assembly of this organelle that must be coordinated also with cell division (Krause and Balish, 2001; Seto et al., 2001).

The avian pathogen *M. gallisepticum* carries also an attachment organelle (Fig. 6) and its cytadherence, like that of *M. pneumoniae*, is a complex multifactorial process involving the coordinate action of a primary cytadhesin molecule GapA and several additional cytadherence-associated proteins in a manner reminiscent of the complex cytadherence mechanism of *M. pneumoniae*. The gene *crmA* (cytadherence-related molecule A), located immediately upstream of *gapA,* has significant sequence homology to the ORF6 of the *M. pneumoniae* P1 operon (Papazisi et al., 2000; Mudahi-Orenstein et al., 2003; Winner et al., 2003) and must be present together with GapA to mediate cyt-

adherence of *M. gallisepticum* (Papazisi et al., 2002a). An *M. gallisepticum* gene encoding a 105-kDa protein, showed 45% homology to the *M. pneumoniae* P1 gene, 46% homology to the *M. genitalium* MgPa adhesin gene, and 47% homology to the *M. pirum* P1-like protein gene (Goh et al., 1998). Along the same line, the P30 adhesin gene of *M. pneumoniae* shows 40.9% identity with the 32-kDa protein gene of *M. gallisepticum*, and 31.4% identity with the *M. genitalium* P32 cytadhesin gene (Hnatow et al., 1998). Collectively, these findings suggest the presence of a family of cytadhesin genes conserved among the pathogenic mycoplasmas belonging to the pneumonia phylogenetic clade. One may speculate that these cytadhesin genes originate from the same ancestral operon.

Mycoplasma hyopneumoniae adheres to the cilia of the respiratory epithelium of swine (Young et al., 2000). The gene coding for the ciliary adhesin was cloned and sequenced, and its 94–97-kDa protein product was characterized (Minion et al., 2000). The R1 region near the carboxy terminus of the protein mediates the adhesion of the mycoplasmas to the cilia. Eight R1 repeating units are required for cilia binding. Interestingly, *M. flocculare*, genetically closely related to *M. hyopneumoniae*, lacks the 97-kDa ciliary cytadhesin gene (Blank and Stemke, 2001), possibly explaining the lack of pathogenicity of this mycoplasma to swine. Cytadherence of *M. hyopneumoniae* causes a fast increase in the intracellular free Ca^{+2} concentration in the infected porcine ciliated tracheal epithelial cells, probably causing damage and loss of the ciliary epithelium (Park et al., 2002).

The receptors identified so far on the host cell membranes responsible for mycoplasma attachment are mostly sialoglycoconjugates and sulfated glycolipids (Razin and Jacob, 1992; Zhang et al., 1994; Kitzerow et al., 1999). The glycoprotein fibronectin, known to serve as an host receptor in the binding of streptococci, staphylococci, mycobacteria, borrelia and treponema appears also to serve as a receptor in the binding of *M. penetrans* (Giron et al., 1996) and of *M. pneumoniae* (Dallo et al., 2002). Somewhat unexpected was the identification of the cytoplasmic elongation factor Tu and the E1β subunit of pyruvate dehydrogenase as the mycoplasmal fibronectin binding proteins. Apparently, the *M. pneumoniae* EF-Tu and the pyruvate dehydrogenase subunit, in addition to their major cytoplasmic biosynthetic and metabolic roles, can be translocated to the cell surface and show multifunctionality. Along this line is the recent identification of glyceraldehyde-3-phosphate dehydrogenase of *M. genitalium* acting, in addition to its important metabolic role, as an adhesin to host cell surface mucin (Alvarez et al., 2003). It is worth mention-
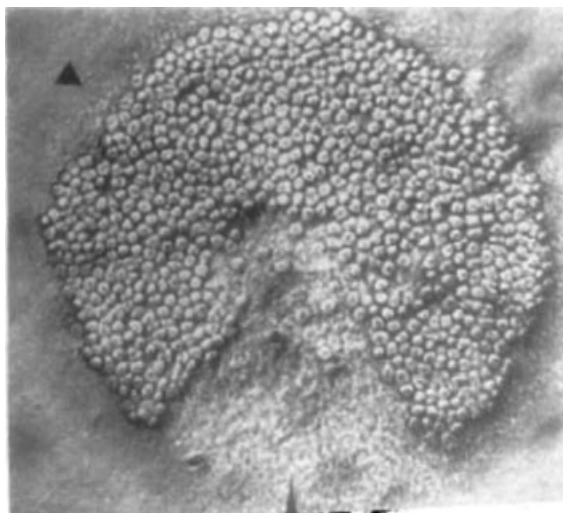
Fig. 22. Selective hemadsorption of human erythrocytes to a distinct sector of a *Mycoplasma gallisepticum* colony. The phase-variable hemagglutinin is not expressed in the colony sector incapable of hemadsorption. From Athamna et al. (1997).

ing that Layh-Schmitt et al. (2000) detected the E1α subunit of pyruvate dehydrogenase as part of a cross-linked complex of the *M. pneumoniae* cytadhesins. What weight is to be given to the fibronectin cytadhesion system when compared to the well-established specific cytadhesin system (P1, P30, etc.) described above? Does the novel system fulfill an auxilliary role in mycoplasmal attachment to host cells? Obviously, these are issues that require further studies.

Not surprisingly, the surface-exposed adhesin proteins are among the most immunodominant mycoplasmal antigens. Furthermore, at least some mycoplasmal adhesins, for example, the Vaa proteins of *M. hominis* (Kitzerow et al., 1999), the Vsp proteins of *M. bovis* (Sachse et al., 2000), the MMA2 protein of *M. arthritidis* (Washburn et al., 1998), the pMSA and pMSB hemagglutinins of *M. synoviae* (Bencina et al., 2001), as well as certain hemagglutinins of *M. gallisepticum* (Boguslavsky et al., 2000; Fig. 22) exhibit the phase- and size-variation properties to be discussed in the next section.

Antigenic Variation

The mycoplasma plasma membrane, being exposed to the external environment, is the cell organelle that comes into contact with the constituents of the host immune system. Lacking the protection of a rigid cell wall, mycoplasmas are particularly sensitive to growth inhibition and lysis by antibodies and complement. Yet, despite

this marked sensitivity, mycoplasma infections are usually chronic in nature, indicating the frequent failure of the host defense mechanisms to eradicate the parasites. Apparently, many pathogenic mycoplasmas possess rather sophisticated mechanisms for rapid adaptation to changing microenvironments. Reversible switching of the expression and modification of major membrane protein antigens on the mycoplasma surface provides an effective escape from rapid destruction by the host immune system (Yogev et al., 2002). Considering the chronic nature of mycoplasmal infections, the constant selection of subpopulations, rather than adaptation of an entire population to an environmental change, is apparently responsible for the heterogenous populations of these organisms.

Table 6 presents the mycoplasmal antigenic variation systems studied thus far. The data enable some generalizations to be made: 1) Most of the systems consist of lipoproteins exposed on the external cell surface and anchored to the membrane via acyl chains. 2) The lipoproteins, functioning as dominant cell antigens, carry repetitive sequences that can spontaneously vary in size through changes in the number of these motifs (size variation). Moreover, the lipoproteins vary not only in size but also may be subjected to "on" and "off" expression states (phase variation). 3) Cloning and sequencing of the lipoprotein genes has revealed that the encoded proteins consist of three regions: the N-terminal leader region, an intermediate domain, and the C-terminal domain composed of reiterated sequences arranged in tandem. Deletion of the leader sequence on processing of the protein leaves a cysteine residue at the N-terminus free for acylation, preferentially by palmitic and myristic acids. The resulting lipid-modified protein is anchored to the outer surface of the membrane through its lipid moiety. 4) In most cases, each mycoplasma strain carries a family of lipoproteins, encoded by multiple variant genes in several versions. The genes are expressed in various combinations and at varying lengths on the organism's surface. Interestingly, in the case of a large family of genes, only one or two genes are expressed at a given time; the others are translationally silent (Bhugra et al., 1995; Glew et al., 1998; Lysnyansky et al., 1999; Sasaki et al., 2002). And 5) in many cases, the surface-exposed lipoproteins function also as adhesins.

The microbial population as a whole may spontaneously and randomly generate distinct cell populations with varied antigenic phenotypes ("heterotypes") that will survive the specific host response capable of eliminating the predominant "homotypes." The frequency of occurrence of such genetic variants is strikingly high (10^{-4} to 10^{-2} per cell per generation,

Table 6. Surface variable antigenic systems in mycoplasmas.

Organism	Genes involved	System components	Mechanism of variation	References
M. hyorhinis	*vlp* gene family	Lipoproteins	Strand slippage (poly A)	Yogev et al., 1991a
M. bovis	*vsp* gene family	Lipoprotein, chimeric lipoprotein	Site-specific inversions homologous recombination	Lysnyansky et al., 2001
M. agalactiae	*avg* gene family	Lipoproteins		Flitman-Tene et al., 2003
M. gallisepticum	*PMGA* (*vlh*) gene family	Lipoproteins	Strand slippage (GAA)	Glew et al., 1998
	pvpA (single copy)	Integral protein (not lipid-modified adhesin)	Point mutation (GAA)	Boguslavsky et al., 2000
M. hominis	*lmp* gene family	Lipoproteins (p120; p135)	Unknown; phase-variable masking	Ladefoged and Christiansen, 1998
	vaa single copy gene	Lipoprotein adhesin	Strand slippage (poly A)	Zhang and Wise, 1997
M. fermentans	P78/ part of an ABC transporter operon	Lipoprotein	Strand slippage (poly A)	Theiss and Wise, 1997
	malp single copy gene	Lipoprotein	Posttranslational modification	Calcutt et al., 1999b
M. penetrans	*mpl* genes (P35, P34A, P34B and P38)	Lipoproteins	Promoter inversion	Horino et al., 2003
M. arthritidis	*Maa2* single copy gene	Lipoprotein adhesin	Strand slippage (poly T)	Washburn et al., 1998
M. mycoides subs. mycoides (SC)	*vmm* gene	Lipoprotein	Strand slippage $(TA)_n$ insert in promoter region	Persson et al., 2002
M. synoviae	*vlh* pseudogene family	Chimeric lipoproteins adhesins	Gene conversion	Noormohamadi et al., 2000
M. pulmonis	*vsa* gene family	Lipoproteins	Site-specific inversions	Shen et al., 2000

compared with 10^{-6} to 10^{-8} of an average mutation).

Lipoproteins may not be the only variable mycoplasma membrane proteins exposed on the cell surface. Thus, the *M. gallisepticum* PvpA is a non-lipid-modified, integral phase-variable membrane protein (Boguslavsky et al., 2000; Yogev et al., 2002). Nevertheless, this mycoplasma carries a highly immunogenic lipoprotein, pMGA, undergoing high-frequency phase variation spontaneously and independently (Glew et al., 1998). There is solid experimental evidence for the occurrence of antigenic variation in vivo. Thus, multiple isolates of *M. hominis* obtained from the same joint of a septic arthritis patient during a six-year period exhibited antigenic variation, although the strains were indistinguishable by restriction endonuclease analysis (Olson et al., 1991). Field infections of sheep and goats by *M. agalactiae* (Flitman-Tene et al., 2000) and chicken by *M. gallisepticum* (Glew et al., 2000) indicate that high-frequency antigenic phase variation occurs in vivo.

The different genetic mechanisms that form the genetic basis for antigenic variation, including strand slippage, homologous recombination, gene conversions, gene duplications, addition or deletion of tandem repetitive units, and DNA inversions (Table 6) are discussed in detail by Razin et al. (1998) and Yogev et al. (2002) and are represented schematically in Fig. 23.

VARIATIONS IN THE *M. PNEUMONIAE* P1 AND P30 ADHESIN GENES A hot spot for frequent mutation within a structural gene was found in the major P1 cytadhesin gene of *M. pneumoniae*. Reversible and spontaneous cytadherence-negative and cytadherence-positive mutants were associated with an insertion or deletion of a single nucleotide in a stretch of seven adenines. This mutation resulted in a frameshift and generation of a termination codon causing the premature termination of P1 translation (Su et al., 1989).

Large portions of the coding regions of the *M. pneumoniae* P1 operon exist as multiple extragenic copies over the chromosome, named "RepMP2/3," "Rep MP4" (originating in the P1 gene), and "Rep MP5" (originating in the ORF6 gene of the P1 operon). Relics of these sequences detected in *M. genitalium*, named "MgPa repeats," revealed strong sequence similarities to the repetitive DNA sequences from *M. pneumoniae* (Himmelreich et al., 1997). Clinical isolates of *M. pneumoniae* were found to exhibit some nucleotide sequence variation in the P1
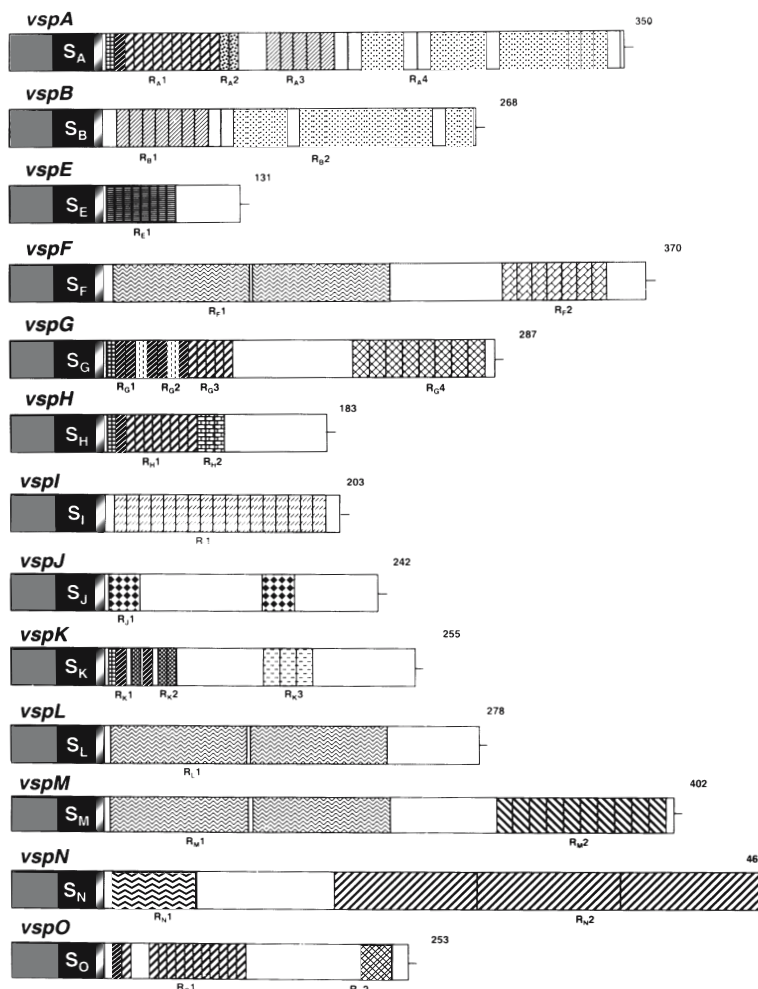
Fig. 23. Schematic representation of regulatory and structural features of antigenic variation systems in mycoplasmas. Representative genes of distinct antigenic variation systems of six mycoplasma species are shown (not to scale) as rectangles consisting of internal blocks aligned from the 5′ end to the 3′ end of each gene. The system designation and the corresponding mycoplasma species are indicated on the left and on the right, respectively. The solid black block, labeled "Signal," contains the species-specific amino acid sequence of a putative lipoprotein signal peptide. Different hatched blocks represent system-specific in-frame reiterated sequences. The location of a homopolymeric tract of contiguous adenines (Poly-A) or of oligo-nucleotide repeats (GAA)n within the promoter region (*vlp*, *pMGA*; Yogev et al., 1991b; Glew et al., 1998) or within the coding gene region (*vaa*, *P78*; Theiss and Wise, 1997; Zhang and Wise, 1997) is shown by an arrow. Two *vsp* genes from two *M. bovis* clonal isolates, exhibiting ON or OFF expression states of the variable surface lipoprotein VspA, are shown. Site-specific DNA inversion during VspA phase variation led to an exchange of the active promoter (VspA-ON) with a non-promoter region (VspA-OFF). The two vspA-upstream regions are differently hatched. Two *vsa* genes isolated from two *M. pulmonis* variants displaying the VsaHA⁻ or the VsaHA⁺ phenotypes are shown (Simmons et al., 1996). A chromosomal fragment that inverted (indicated by "DNA Inversion") during phase transition (VsaHA⁻ ↔ VsaHA⁺) also is indicated by brackets. The direction of expression of the *vsa* gene from each variant is marked by an arrow. From Razin et al. (1998).

gene (Dorigo-Zetsma et al., 2000, 2001). The variable regions in the gene were found to correspond to regions of some of the repetitive elements, suggesting that the P1 gene may vary because of recombination between repetitive regions (Su et al., 1993; Kenri et al., 1999). Accordingly, the *M. pneumoniae* strains were divided into two groups, I and II (Su et al., 1993).

The 30-kDa (P30) adhesin protein of *M. pneumoniae* appears also to undergo size variation, losing proline-rich repetitive sequences in its C-terminal region. The truncated adhesin showed reduced antigenicity and adhesion capacity. The mechanism underlying the loss of the repeated sequences is not clear, but slipped base-pairing during replication or homologous recombination processes between repeated sequences might be

the cause of size polymorphism (Layh-Schmitt et al., 1997).

Immune System Modulation

The complex network of interactions between mycoplasmas and their host immune system involves mycoplasma-induced specific and non-specific immune reactions. Specific protective defense mechanisms include the production of systemic as well as local anti-mycoplasmal antibodies of different classes and subclasses, stimulation of cell-mediated immunity, and opsonization and phagocytosis of organisms. In some cases, for macrophages to kill invading mycoplasmas, some accessory factors provided by the host are required. Thus, alveolar macrophages (serving as the primary effector cells in early *M. pulmonis* killing in infected mice) depend on the presence in the lungs of the collectin (collagen-like lectin) surfactant protein A. Nitric oxide and superoxide radicals produced by the macrophage contribute to the killing either directly or via the production of peroxynitrite (ONOO⁻; Hickman-Davis et al., 1999).

The specific reactions elicited by invading mycoplasmas, essential for resistance and protection against mycoplasma infections, also have been shown to play a role in the development of lesions and exacerbation of mycoplasma-induced diseases (Razin et al., 1998). Humoral immunity has a major role in defense against systemic dissemination of mycoplasmal infection, but cellular immune responses may be important in exacerbation of mycoplasmal lung disease. Lack of adaptive response in severe combined immunodeficient (SCID) mice infected with *M. pulmonis* was associated with reduced lung lesion severity and with increased mycoplasmal colonization and disease at extrapulmonary sites. The transfer of naïve spleen cells from immunocompetent mice to *M. pulmonis*-infected SCID mice restored the severity of lung lesions, whereas the transfer of convalescent anti-*M. pulmonis* serum to the infected SCID mice did not restore lung lesion severity but prevented arthritis (Cartner et al., 1998).

Although mycoplasmas circumvent phagocytosis (Marshall et al., 1995; Minion, 2002), they appear to interact with mononuclear and polymorphonuclear phagocytes, suppressing or stimulating them by a combination of direct and indirect cytokine-mediated effects. For extensive recent reviews on cytokine induction by mycoplasmas, the reader is referred to Razin et al. (1998) and to Rawadi and Roman-Roman (1998). These immunomodulatory influences depend on both the immune cells and on the mycoplasma species involved. Thus, *M. dispar* manages to bypass phagocytosis by bovine alveolar macrophages because it is covered by a polysaccharide capsule which inhibits activation of macrophages and suppresses the production of tumor necrosis factor (TNF)-α and interleukin (IL-1; Almeida et al., 1992). Other mycoplasmas activate monocytes, macrophages and brain astrocytes and induce secretion of the proinflammatory cytokines TNF-α, IL-1 and IL-6, chemokines (such as IL-8, monocyte chemoattractant protein 1, macrophage inflammatory protein 1αØβ, and granulocyte-monocyte colony-stimulating factors [GM-CSFs] and prostaglandins), and active oxygen and nitrogen metabolites (Razin et al., 1998; Ribero-Dias et al., 1999; Yirmiya et al., 1999).

In addition to eliciting antimycoplasmal immune responses, mycoplasmas exert a wide range of nonspecific immunomodulatory effects upon cells comprising the immune apparatus (Reyes et al., 1999). Mycoplasmas have been shown to affect the immune system by inducing either suppression, or polyclonal stimulation of B and T lymphocytes, induction of cytokines, increasing cytotoxicity of macrophages, natural killer cells and T cells, enhancing expression of cell receptors and activation of the complement cascade. The ability of mycoplasmas to immunomodulate host immune responsiveness contributes to their pathogenic properties, enabling them to evade or suppress their host defense mechanisms and establish a chronic, persistent infection (reviewed in Razin et al. [1998]). The cytokine network plays a critical role in the maintenance of immune system homeostasis. The overproduction of some cytokines in response to mycoplasma infections may upset the fragile equilibrium and thereby contribute to triggering or aggravating autoimmune diseases (Rawadi and Roman-Roman, 1998).

Mycoplasmas are able to stimulate as well as suppress lymphocytes in a nonspecific, polyclonal manner, both in vitro and in vivo. Apparently, mycoplasmal B cell mitogens differ from bacterial lipopolysaccarides (LPS) in that they are able to activate lymphocytes from C3H/HeJ mice, which respond poorly to LPS, and their inducing potential is unaffected by polymyxin (Brenner et al., 1997). Modulation of monocyte-macrophage activity by mycoplasmas has been demonstrated in vitro by augmented TNF-α-mediated tumoricidal activity towards different tumor cell lines (Razin et al., 1998). The proven capacity of many mycoplasmas to trigger the production of proinflammatory cytokines in vivo may account for much of the pathology observed during mycoplasma infections and might also influence the subsequent interactions of these microorganisms with lymphoid cells (Manimtim et al., 2001). In addition to affecting various subsets of lymphocytes, mycoplasmas and myco-

plasma-derived cell components modulate the activities of monocytes-macrophages and natural killer (NK) cells and trigger the production of a wide variety of up- and downregulating cytokines and chemokines (Razin et al., 1998; Rawadi and Roman-Roman, 1998). Mycoplasma-mediated secretion of proinflammatory cytokines (such as TNF-α, IL-1 and IL-6) by macrophages and of upregulating cytokines by mitogenically stimulated lymphocytes play a major role in mycoplasma-induced immune modulation and inflammatory responses. On the other hand, the ability of some mycoplasmas to induce downregulating cytokines (such as IL-10, IL-13 and possibly also transforming growth factor [TGF-β]) may contribute to their ability to evade host immune mechanisms by perturbation of macrophage accessory and phagocytic functions. In this way, decreasing production of proinflammatory cytokines and T cell proliferation affect the balance between different T helper cell subsets. The potential to alternately stimulate or suppress the immune system would impart a distinct advantage to any pathogen attempting to survive in the hostile and changing environment of the infected host.

As to the mechanism of cytokine induction by mycoplasmas, Rawadi et al. (1998) reported that protein tyrosine kinase is absolutely required by the *M. fermentans* lipoproteins (LAMPf) to induce proinflammatory cytokines in monocytic cells. The mycoplasmal membrane lipoproteins bind to a cellular receptor(s) that mediates directly or indirectly protein tyrosine kinase activation. This binding triggers the activation of several signal cascade pathways that lead to the phosphorylation and translocation of transcription factors into the nucleus, resulting in transcription and translation of cytokine mRNAs.

Apparently, certain mycoplasmas have more than one cell component with mitogenic activity, and mycoplasmal mitogens are not necessarily identical to mycoplasmal cytokine-inducing elements. *Mycoplasma penetrans* appears to possess several T or B cell mitogens (Brenner et al., 1996; Feng and Lo, 1999), including a glycolipid and a major membrane lipoprotein. However, although the mitogenic *M. penetrans* lipoprotein is also highly immunogenic in *M. penetrans*-positive AIDS patients and elicits production of specific antibodies (Lo, 1992; Brenner et al., 1996), the glycolipid is not recognized in sera of *M. penetrans*-infected individuals.

Of special interest as immunomodulators are the mycoplasmal lipopeptides (MALPs) which resemble the classical murein lipoprotein in that they carry a fatty acid-substituted N-terminal S-(2,3-bis-acyclo-oxypropyl) cysteinyl group but lack the N-acyl long chain fatty acid of the classical bacterial lipoproteins (Muhlradt et al.,1997;

Muhlradt, 2002). This feature renders these mycoplasmal compounds exceptionally active in vitro as macrophage stimulators (Deiters and Muhlradt, 1999). Microgram quantities of the *M. fermentans* MALP-2 were found to stimulate the in vitro as well as in vivo release of chemokines from macrophages, being more potent than LPS on a weight basis (Deiters and Muhlradt, 1999). The co-administration of MALP-2 with a model antigen resulted in potent enhancement of humoral and cellular antigen-specific immune responses, suggesting the use of the lipopeptide as a promising adjuvant for delivery of vaccine antigens (Rharbaoul et al., 2002). Biogenesis of the MALP-2 lipopeptide from the full-length *M. fermentans* lipoprotein MALP-404 involves the extracellular, site-specific proteolytic processing of the lipoprotein yielding the MALP-2 surface-bound lipopeptide as well as a stable, released fragment of MALP-404. This defines a new mechanism of secretion in mycoplasmas and a novel means of altering the phenotype of mycoplasmas through posttranslational processing (Davis and Wise, 2002). Of phylogenetic interest is the finding that the above mycoplasmal lipoproteins share conserved amino acid motifs with lipoproteins of *B. subtilis*, *Treponema pallidum* and the relapsing fever *Borrelia* spp. (Calcutt et al., 1999b; Rosati et al., 1999). In summation, the above data indicate that mycoplasmal lipoproteins and lipopeptides are probably the most relevant mycoplasmal components in the early host reaction. The primary target cells are likely to be the alveolar macrophages liberating chemokines that attract further leukocytes. The MALP-2 mediated response is dependent on the signaling molecules Toll-like receptor 2 (TLR2) and the activation of caspases, inducing apoptosis and cell necrosis (Rawadi, 2000; Luhrmann et al., 2002; Into et al., 2002).

Another chemically defined immunomodulator, produced by *M. arthritidis,* is MAM, a soluble excreted protein of 26kDa acting as a superantigen (reviewed in Cole et al. [2000] and Washburn and Cole [2002]). MAM activates a major fraction of murine T cells and a minor fraction of human T cells by crosslinking MHC class II to TCR, primarily through zinc-dependent binding to the MHC class II E alpha chain in the mouse and to the DR alpha chain in man (Langlois et al., 2003). Activation of T cells (mainly Vβ6 and Vβ8 TCR subsets in mice and Vβ17 and Vβ3 subsets in humans) induces clonal proliferation and release of lymphokines, including IL-2, IL-4, IL-6 and interferon and nitric oxide (Ribero-Dias et al., 2003). Of note is that MAM is the only mycoplasmal superantigen defined thus far. The finding of antibodies to MAM or a portion of the MAM molecule in the sera of rheumatoid arthritis (RA) patients raises

Table 7. Some mycoplasmal cell components active in immunomodulation.

Component	*Mycoplasma*	Biological activity	References
MAM (27-kDa basic protein)	*M. arthritidis*	Superantigen, activating T cells	Cole et al., 2000 Washburn and Cole, 2002
Lipopeptide (MALP)	*M. fermentans*	Activating macrophages, stimulating cytokine and chemokine release (IL-1, IL-6 and TNF-α)	Deiters and Muhlradt, 1999 Muhlradt, 2002
Phosphocholine-containing phosphoglycolipids	*M. fermentans*	Fusogenic agents with cytokine induction activity	Rottem, 2002

Abbreviations: MAM, *M. arthritidis* mitogen; MALP, macrophage-activating lipopeptide; IL, interleukin; and TNF, tumor necrosis factor.

the possibility that MAM or MAM-like epitopes crossreactive with MAM may play a role in RA (Cole et al., 2000), but this clearly requires further study before any definite conclusions can be drawn.

Except for the chemically characterized *M. arthritidis* superantigen MAM, the macrophage-stimulating lipopeptides, and the phosphocholine-containing phosphoglycolipid of *M. fermentans* (Table 7), cell components exhibiting mitogenic and cytokine-inducing activities from the other mycoplasmas need to be identified and isolated. Likewise, there is very little information on the nature of host cell receptors for the mycoplasmal immune modulators.

Applications

The theoretical contributions derived from the application of mycoplasmas as effective research models applied to solve basic problems in biology, including biomembrane and genomic research, have been discussed at length in many sections of this chapter. On the technological side, most of the applications emanating from the molecular biology and genetic research of mycoplasmas concern the development of diagnostic kits and DNA vaccines, described in preceding sections of this chapter. While discussing biotechnological applications, mention should be made of a negative aspect resulting from the frequent troublesome infection of cell cultures by mycoplasmas (see the section Habitats and Ecology). According to regulations of the United States Food and Drug Administration, every biological product produced in cell cultures should be tested for the presence of mycoplasmas, an expensive step that cannot and should not be avoided.

The high cost of mycoplasma cultivation and low yields of organisms do not encourage their use as a source for production of biochemicals, unless the material is unique. This seems to be the case for the peculiar DNA methylase produced by *Spiroplasma monobiae* (MQ-1). The enzyme was found in our laboratory (Nur et al., 1985) to methylate all cytosine residues located at the sequence cytosine-guanine, the only sequence methylated in eukaryotic DNA. To exploit this finding, the methylase gene of the spiroplasma was cloned, and a special system was developed to express it in *E. coli* to overcome the UGA problem (see the section Codon Usage). Expression in *E. coli* could be used to produce large quantities of the enzyme on a commercial basis needed as a research reagent in laboratories working on eukaryotic DNA methylation.

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CHAPTER 1.2.30

The Phytopathogenic Spiroplasmas

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Introduction

Spiroplasmas (family Spiroplasmataceae, order Mycoplasmatales) comprise a group of helical prokaryotes whose external morphology, unlike that of the spirochetes, is defined by an internal skeletal framework rather than by an external peptidoglycan-containing cell wall. The loss of a cell wall in mollicutes (spiroplasmas, mycoplasmas, phytoplasmas, acholeplasmas and others) is due to degenerative evolution from their walled precursors (see also The Phytopathogenic Spiroplasma in this Volume, which provides extensive coverage of this taxon). Most of the more than 50 spiroplasma species that have been described are associated with insects and other animals in relationships that range from pathogenic to benign. For example, *Spiroplasma melliferum* infects honeybees (Whitcomb and Williamson, 1979), other spiroplasmas cause disease in mosquitoes (Humphery-Smith et al., 1991), and the "sex ratio" spiroplasma kills the male progeny of fruit flies (Williamson et al., 1989). Many spiroplasmas, however, colonize the gut lumen and/or hemolymph of their insect hosts without causing detrimental effects (Whitcomb and Williamson, 1979). Only three species of spiroplasmas (*S. citri*, *S. kunkelii* and *S. phoeniceum*) are known to cause disease in plants. These microbes are important for two reasons. First, they cause significant losses in economically important crops such as maize (corn stunt disease) and citrus (citrus stubborn disease), and in specialty crops (brittle root of horseradish). Second, and perhaps just as important, the plant pathogenic spiroplasmas, which are cultivable in artificial media, serve as models for the more numerous, widespread, and noncultivable phytoplasmas, a group of related mollicutes that cause disease in over 300 plant species globally. In this chapter, the focus is on the unique features and issues related to the small, but economically important, group of spiroplasmas that cause diseases in plant hosts.

Historical Background

For many years, the diseases citrus stubborn and corn stunt were attributed to viral etiology, as their agents were graft transmissible and could not be cultivated. Remission of symptoms in stubborn diseased citrus trees following application of tetracyclines, but not penicillin (Igwegbe and Calavan, 1970), suggested that a nonviral agent might be involved. But a novel microbial taxon was not recognized until the late 1960s when thin sections of maize displaying strong symptoms of a long-known, economically serious stunting and yellowing disease called "corn stunt" were examined by electron microscopy (Davis and Worley, 1973). The unusual prokaryotes visualized in those sections (later designated "spiroplasmas") were smaller than most bacteria and had a distinctive helical morphology and lacked the peptidoglycan-rich outer wall that defines most prokaryotes.

Morphology

The first descriptions of the morphology of spiroplasmas were made by Davis and colleagues (Davis et al., 1972; Davis et al., 1973), who used phase optics to view sap expressed from corn plants with symptoms of corn stunt disease. They described the associated microbes as helical filaments about 3–15 μm long and 200–250 nm in diameter with a regular wave (gyre) length and an amplitude of 0.4 μm. Later designated "*S. kunkelii*," this microbe was recognized as a new taxon. An early description of *S. citri* indicated that it was somewhat larger at 410–500 nm diameter (Liu, 1981). Some helical spiroplasmas were accompanied by spherical bodies of 400–600 nm diameter (Davis and Worley, 1973; De Leeuw et al., 1985); when grown on agar for several days, older spiroplasmas became elongated, branched, and even appeared to split into beaded chains (De Leeuw et al., 1985). With electron microscopy the spiroplasma outer membrane was

clearly free from a surrounding wall and organized as a single triple-layered unit whose thickness ranged from about 7.0–7.5 to about 10 nm (Davis and Worley, 1973; Liu, 1981). The presence of cholesterol in spiroplasma membranes, an unusual feature in bacteria, presumably adds rigidity to this structure. No visible flagella or other structures that could explain the observed motility were found. The two termini of the spiroplasma helix are usually different, one being rounded or blunt and the other narrowing to a point (Garnier et al., 1981). A recent electron microscopy study by Ammar et al. (2004) provided new details on spiroplasma morphology. Helix lengths of four spiroplasma species (the plant pathogens *S. citri* and *S. kunkelii* and the non-phytopathogens *S. floricola* and *S. melliferum*) varied among the species. A globular section was often seen at one end, and small rounded structures (possibly the same as the "spherical bodies" described earlier by Davis and colleagues) appeared to bud from the main helix.

Spiroplasmas grown in culture may occur in clusters or groups. Townsend et al. (1980) described commonly occurring "medusa-like aggregates" of helical filaments extending from a central point. Such aggregates apparently result from a failure of spiroplasmas to separate following cell division.

How spiroplasmas maintain their unique helical morphology (Fig. 1) in the absence of a cell wall, such as that of spirochaetes, and how they move in the variety of ways observed without visible motility-related appendages such as flagella, were questions of great interest in the early days of spiroplasma characterization. Internal fibrils were observed in several spiroplasma species, including the plant pathogen *S. citri,* using various electron microscopic and other

Fig. 1. *Spiroplasma citri* in liquid culture, shown with dark field optics. Photo: G. Baker and J. Fletcher.

strategies (Cole et al., 1973; Williamson and Whitcomb, 1974b; Williamson et al., 1974; Razin, 1978; Stalheim et al., 1980; Townsend et al., 1980; Charbonneau and Ghiorse, 1984), and actin-like proteins were identified in several strains of *S. citri* (Kirchoff, 1992). Taking advantage of recent technological advances, Trachtenberg and colleagues (Trachtenberg et al., 1987; Trachtenberg et al., 2003; Trachtenberg, 1998; Trachtenberg and Gilad, 2001) have provided a more complete understanding of spiroplasma morphology. The picture emerging is that of the spiroplasma cell as a tubular structure, inside which a flat ribbon, about 940 Å wide and composed of parallel fibrils, each 45–50 Å in diameter, is attached along its length to the tube's inner wall (the cell membrane). Tube and ribbon together assume a helical shape such that the ribbon extends down the shortest side of the tube. The major fibril protein, encoded by the *fib* gene (Williamson et al., 1991), probably consists of a tetramer of 59-kDa monomers. Multiple tetramers associate to form the fibrils, and seven fibrils adjoin in a single flat ribbon (Trachtenberg and Gilad, 2001). Fibrils in different spiroplasma species are similar, as demonstrated by the reaction of antibodies made against *S. melliferum* BC3 fibrils with fibrils from 13 other groups (Townsend and Archer, 1983). The fibrillar skeleton has been suggested to function like a linear motor, in which coordinated shortening and lengthening caused by conformational changes in the tetramer proteins determine shape and motion (Trachtenberg and Gilad, 2001).

Some mycoplasma pathogens of animals and humans have been described as flask-shaped, with the "neck" of the flask comprising an attachment organelle (tip structure) involved in adherence. Liu and colleagues (Liu, 1981; Liu et al., 1983) described a long, thin, electron-dense neck for *S. citri* cells during their residence in the intestinal tract of the leafhopper vector, *Circulifer tenellus*, and postulated that it might aid in penetration of the endoplasmic reticulum, with which these authors often found spiroplasmas associated. Phillips and Humphery-Smith (1995), studying a mosquito-pathogenic spiroplasma, *S. taiwanense,* also described maturing spiroplasmas as pleomorphic and presented transmission electron micrographs showing rounded cells with narrower extensions consistent with a neck structure. Recent ultrastructural studies of *S. kunkelii* were consistent with Liu's and Humphery-Smith's observations; Ammar et al. (2004) and Ozbek et al. (2003) reported that the cores of the *S. kunkelii* helix termini (the pointed or rounded ends that result following cell division) were more electron-dense than those of the central regions of the cell. *Spiroplasma kunkelii* cells consistently aligned themselves tip-down among

the midgut microvilli, and the penetration of the tips within the lamina densa suggested a role for the tip structure in adhesion, similar to that of the attachment organelles of animal pathogenic mycoplasmas, and in traversal of the basal lamina, a specialized extracellular matrix (containing laminin, nidogen and collagen; Sreebny, 1988) that functions as a selective barrier and plays a role in regulating various cell functions. *Spiroplasma citri* cells also attach via their ends to the lumen-side surface of gut epithelial cells prior to invasion (Kwon et al., 1999), but morphologically distinct tip structures were not observed in that study (A. Wayadande and J. Fletcher, unpublished observations). As with zoopathogenic mycoplasmas, a flask-shaped tip could contribute to another type of spiroplasma motility, termed "gliding," in which the cells travel in the direction of their tips.

Although helical cell morphology is one of the most distinctive features of spiroplasmas during their life in the plant and also in medium, it is not always maintained in the alternate host/insect vector. Townsend et al. (1977) noted that immediately after microinjection into the hemolymph of the experimental leafhopper vector, *Euscelidius variegatus*, many helical cells of *S. citri* could be seen by electron microscopy, but over the period of a week, the helices gradually disappeared and were replaced by tiny, pleomorphic bodies. Loss of helicity in the insect was noted also by Liu (1981), who described *S. citri* cells in the vector *C. tenellus* as consisting of a main body with a long, thin, electron-dense branch, and by Kwon et al. (1999). Interestingly, although *S. kunkelii* apparently loses helicity in its vector, the corn leafhopper *Dalbulus maidis* (Granados and Meehan, 1975), it remains helical in other leafhopper species including *D. elimatus* and *E. variegatus* (Markham et al., 1977). That spiroplasmas could be cultivated from such hemolymph despite the absence of helical cells supported the conclusion that the pleomorphic bodies were, in fact, viable spiroplasmas. In a natural setting, spiroplasmas reach the hemocoel of their insect vectors only after traversal of the gut wall. In such cases the loss of helicity may occur before the microbes reach the hemocoel. In recent work (Ozbek et al., 2003; Ammar et al., 2004), cross sections of *S. kunkelii* cells that had invaded midgut epithelial cells were examined; spiroplasmas located near the gut lumen side of the epithelial cell were of normal size while those residing on the hemolymph side of the epithelial cell were significantly larger, suggesting that the change from a helical to a pleomorphic morphology occurred during the journey across the epithelial cell cytoplasm.

Recent transmission electron microscopy (Ozbek et al., 2003) of *S. kunkelii* cells within the bodies of *D. maidis* revealed surface appendages interpreted as fimbriae and pili. The former structures are long, narrow (10–12 × 211–1555 nm) organelles that, in other bacteria, contribute to adherence (to host cells or to other bacteria) and also may participate in cell signaling. The apparent contact between the *S. kunkelii* fimbriae, arranged peritrichously, and the vector insect's gut epithelial and muscle cell outer surfaces is consistent with the concept of a similar role in attachment for spiroplasma fimbriae. Indeed, the authors suggest roles for them also in detachment of host cell laminae from the underlying cell layer and prevention of host cell apoptosis. Shorter, polar-located filaments resembling type IV pili of many other bacteria also were described in *S. kunkelii* (Ozbek et al., 2003; Ammar et al., 2004; Bai et al., 2004). Type IV pili contribute to many cell functions including "twitching" motility, transformation, virus infection, biofilm formation, activation of host responses and bacterial conjugation. Proteins of the traE group are commonly implicated in pilus formation; interestingly, four *traE* genes recently have been identified in *S. kunkelii* (Bai et al., 2004). Other surface-associated structures include rod-shaped spiroplasma viruses (Fig. 2), approximately 230–280 by 10–15 μm in size (Bové et al., 1989). Viruses, too, adhere at their tips, perpendicularly, to the mollicute membrane (Townsend et al., 1977; Sha et al., 1993), such that the particles may appear very similar to those structures described as pili or fimbriae.

When diluted suspensions of spiroplasmas are spread onto the surface of appropriate agar-solidified media they produce slow-growing colonies. Although mycoplasmas are known for their distinctive "fried egg" colony morphology, spiroplasmas colonies typically are not so described. Their tiny colonies are usually characterized by indistinct edges resulting from a halo of satellite colonies, produced by spiroplasmas
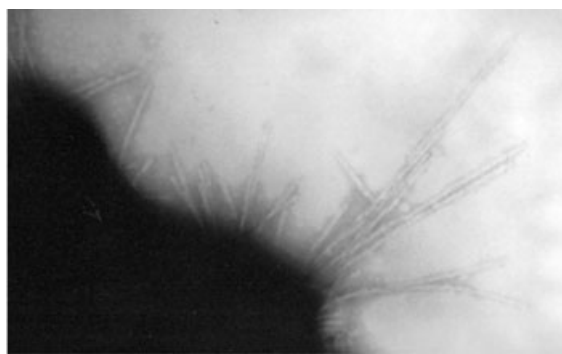
Fig. 2. Electron micrograph of spiroplasma virus SVTS2 on the surface of *S. citri* M200H cell. Photo: Y.-H. Sha and J. Fletcher.
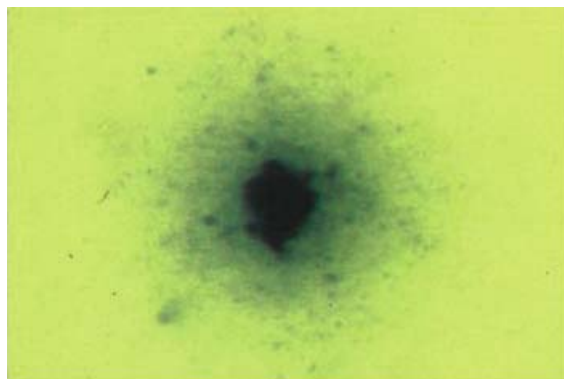
Fig. 3. Colonies of *S. citri* BR3-3X on agar-solidified medium. Photo: J. Fletcher.

that have traveled through the pores in the medium (Fig. 3).

Motility

Davis and Worley (1973) described spiroplasmas as cells that "whirled or spun rapidly about the long axis" and exhibited distinctive "vermiform" movements including flexing, folding and curling as well as contractile movements that progressed in a wave from one end of the cell to the other. Motility is greatest when spiroplasmas are grown under optimal conditions. In dense substrates spiroplasmas also display translational movement and are sensitive to chemical gradients, responding to various compounds with either positive or negative chemotaxis (Daniels et al., 1980). The role of motility in colonization of host tissues in the plant or insect vector is not well understood, but it may be a factor in the accumulation of spiroplasmas in growing plant terminals and in the salivary glands of insects.

Loss of helicity and motility seems to occur naturally at a low rate. A naturally-occurring nonhelical and nonmotile strain, ASP-1, of *S. citri* was isolated serendipitously within a population of normally helical cells from "little-leaf" affected orange trees (Townsend et al., 1977). The novelty of the strain was recognized initially by the unusual colony morphology. The lack of normal motility (ASP-1 cells displayed only a movement described as "twitching") resulted in colonies having sharply defined edges. Protein profiles revealed the absence, in ASP-1, of a single polypeptide of 39 kDa. Neither the protein, nor the helicity and motility, were necessary for pathogenicity, as the natural mutants produced symptoms indistinguishable from those of wildtype following the confinement of ASP-1-injected leafhoppers onto test plants. A few years later, Lee and Davis (1987) isolated several nat-

urally occurring variants of *S. kunkelii* having varying degrees of defects in helicity and motility. One strain having normal helical cells displayed little or no motility, indicating that the two features were independent of one another.

Recently, motility of *S. citri* strain GII3 was interrupted by Tn*4001* mutagenesis (Foissac et al., 1997). The inactivated gene, identified and designated "*scm1*," could be complemented with the wildtype gene (Jacob et al., 1997), suggesting a critical role for the *scm* gene product in spiroplasma motility.

Cell Division

As far as is known (see The Genus Spiroplasma in the second edition) the general outlines of cell division of phytopathogenic spiroplasmas are the same as those of other mollicutes. However, because spiroplasma morphology is different from that of other mollicutes there are some unique aspects. For example, the helicity of spiroplasmas provides a simple meter stick by which to measure length requirements for division. Garnier et al. (1981) reported that the minimum length of *S. citri* cells grown in culture was two complete helices. In an actively growing culture, cell division occurred by constriction near the center, usually of spiroplasmas having four complete turns, so that both products of division would be of the minimal size. Growth at higher temperatures (37°C) prevents cell division, resulting in elongated cells consisting of multiple turns.

Growth is necessary for cell division. Where that growth occurs is difficult to assess because of conflicting results (Garnier et al., 1984). Antibody labeling suggests that growth of the membrane is diffuse throughout the length of the cell. However, labeling with tritiated amino acids or of redox sites with tellurite suggests polar growth.

Models accounting for cell shape based on the structure and activity of fibril protein-containing structures invoke a role for the structures in DNA segregation (Townsend, 1983). Such fibril-composed structures, and their roles in spiroplasma morphology, have been more completely described in recent work (Trachtenberg et al., 1987; Trachtenberg, 1998; Trachtenberg and Gilad, 2001; Trachtenberg et al., 2003; see the section Motility above in this Chapter).

Zhao et al. (2004), working from the genome sequence of *S. kunkelii* (see the section Strains, Species and Taxonomic groups below in this Chapter) have identified homologues for *ftsZ* and *ftsA* genes in this phytopathogen. The former, in other bacteria, is known to be responsible for the formation of the septum between the two daughter cells.

Phylogeny and Taxonomy

Only three species of plant pathogenic spiroplasmas have been identified to date. New species of spiroplasmas are still being discovered and proposed. However, the rate of discovery is far less than originally predicted from the observation that one in ten species of arthropod examined contained a hitherto undescribed spiroplasma (Hackett and Clark, 1989). Many species have since been identified that are found in, and can grow in, multiple arthropod species. Thus, the *Spiroplasma* species is unlikely to be the most speciose on earth (Gasparich, 2002). Nevertheless, apparently novel species are still being described (Fukatsu et al., 2001), which makes it likely that more than the approximately 50 spiroplasma species known today will eventually be recognized. On the other hand, that all three plant pathogenic species were identified early during the exploration of the genus suggests that there may not be many additional plant pathogenic species.

Strains, Species and Taxonomic Groups

Partial serological crossreaction placed the three phytopathogenic spiroplasmas in group I, with five others (Gasparich, 2002). Each of the three constitutes a separate subgroup within the group: subgroups I-1, I-3 and I-8 corresponding, respectively, to *S. citri*, *S. kunkelii* and *S. phoeniceum*. Phylogenetic differentiation below the subgroup level has been reported between Old World and New World *S. citri* (Igwegbe et al., 1979). The subgroups were established according to the rule that reciprocal DNA-DNA hybridization experiments reveal only 30–70% crossreactivity (Gasparich, 2002). For example, less than 55% crossreaction was observed between *S. citri* and *S. kunkelii* (Lee and Davis, 1980). The 70% crossreactivity value has also been established as the minimum between isolates of bacterial species. Thus, the subgroup designation is equivalent to the species designation. Protein profiles also differentiate group I spiroplasmas into subgroups (Mouches et al., 1982); for example, distinguishing *S. citri* from *S. kunkelii*. Differentiation based on protein profiles, however, is less definitive for other subgroups (Mouches et al., 1983b).

The less than 70% reciprocal DNA-DNA hybridization measuring stick for classification of a pair of isolates into separate species has two theoretical components: the fraction of sequence in one isolate that has homologous sequence in the other and the fraction of that DNA that has retained sufficient nucleotide sequence identity to form stable hybrids with its homolog. The hybridization value does not measure directly the degree of sequence divergence. The availability of sequences of gene-rich regions from both *S. citri* and *S. kunkelii*, however, allows direct estimation of the degree of divergence between the two genomes (U. Melcher and J. Fletcher, unpublished observation). In 39 kbp of compared sequence, only 7.3% was too far diverged to be recognized as homologous. Of the remaining sequence positions, an average of 93.3% (range 88–95%) had identical nucleotides. The high percentage identity should be sufficient to form stable hybrids if hybridization is performed at 15 or more degrees below the melting temperature.

Resolution of the apparent conflict between the close identity in nucleotide sequence for the selected genes and the less than 55% cross-hybridization value should be resolved when genome sequence information for the two spiroplasmas is available. Although a project to sequence the genome of *S. citri* GII3 has been underway (Foissac et al., 2004), the sequence is not yet publicly available. In a different initiative, the *S. kunkelii* genome sequence was almost completely sequenced and the information is posted (http://www.genome.ou.edu/spiro.html {Spiroplasma kunkelii Strain CR2-3x Genome Sequencing Web site}) but not yet fully annotated. Nevertheless, some guesses can be made. Most of the genes contributing to the analysis encode enzymatic proteins essential for the survival of these spiroplasmas in the environment in which they thrive. They are thus under strong negative selection, so that only rare changes are expected. On the other hand, the *spi* genes, encoding a nonenzymatic major surface protein, are known to vary more substantially, even undergoing positive selection for amino acid changes (Foissac et al., 1996; Melcher and Fletcher, 1999a). Thus, a comparison of *spi* nucleotide sequence conservation between *S. citri* and *S. kunkelii* reveals only 84.7% identity with only 68% coverage of the gene. This level is likely too low to produce DNA-DNA cross-hybridization. For comparison, conservation of *spi* between *S. citri* strains is 97–99%, with complete or close to complete coverage. One possible extrapolation of these findings is that the genomes of *S. citri* and *S. kunkelii* consist of no more than 60% of conserved genes, the "core" genome (Hacker and Carniel, 2001) necessary for essential enzymatic functions, and about 40% of highly variable genes, the "flexible" genome, probably encoding surface proteins that interact with the environments of the spiroplasma. An alternate possibility, that *S. citri* and *S. kunkelii* have different complements of mobile genetic elements, is discounted by the observation that the elements frequently encountered in *S. citri*

are also the most frequently found in the *S. kunkelii* genome, accounting for as much as 18% of the genome.

Host Associations

The other five members of group I are not known to be associated with plant disease, though most have some association with plants. For most spiroplasmas, this association is an external one, with the organisms being found in places such as nectaries and leaf and flower surfaces. Plant association is not a unique property of group I spiroplasmas, nor are they associated preferentially with one kind of arthropod; leafhoppers, honeybees, ticks, fruit flies and beetles are among the animal hosts (Gasparich, 2002). Yet, only group I has phytopathogenic members, suggesting that the group has acquired or developed a gene or gene systems allowing colonization of plant interiors. The plant contact could have provided the theater for phytopathogenicity to evolve separately three times in this group. Alternatively, *S. citri*, *S. kunkelii* and *S. phoeniceum* could have derived from a phytopathogenic common ancestor not shared by other group I species.

Evidence suggests that plant pathogenicity did not simply arise once in the common ancestor of the three spiroplasma phytopathogens. In this regard, 16S rDNA sequences are uninformative since the *S. phoeniceum* sequence is missing and identities between the others (98–99%) are too close to be sure of the significance of differences. Though five phylogenetically informative positions cluster *S. kunkelii*, *S. citri* and *S. melliferum* (the latter of which is non-phytopathogenic), only one each suggests that the first two and the last two are closer to one another. Fortunately, the more diverged *spi* gene provides good support for sister status for *S. citri* with *S. melliferum* and for *S. kunkelii* with *S. phoeniceum*. The former pair is 94.2% identical over 99% of their common length and the second pair is 92.6% identical over 98% of the length, as opposed to the much lower cross-pair values for *S. citri* and *S. kunkelii* mentioned above. This result suggests an initial divergence of clades containing an *S. citri-melliferum* ancestor and an *S. kunkelii-phoeniceum* ancestor. In this scenario, plant pathogenicity may have arisen twice, once before the *S. kunkelii-phoeniceum* divergence and once after the *S. citri-melliferum* divergence. Alternatively, both ancestors may have been phytopathogens, with this trait later being lost from *S. melliferum*. Also possible is that pathogenicity of the latter exists but has just not been documented.

Spiroplasma Position Among Mollicutes

To address the question of whether plant pathogenicity may have arisen in other spiroplasma lineages, it is important to review current understanding of Mollicutes evolution (Weisburg et al., 1989; Maniloff, 1992; Williamson et al., 1998; Gasparich, 2002; Gasparich et al., 2004). Early theories that mollicutes were the result of degeneration of L-forms of many different bacteria, or that mollicutes were the ancestors of walled bacteria have been largely discounted. Nevertheless, there is some support for these views. The mollicute *Asteroleplasma anaerobium* appears to be more closely related to genera of Gram-negative bacteria than to other mollicutes (Weisburg, 1989). This observation suggests the Mollicutes class is phylogenetically polyphyletic. Support for a mollicute origin for some walled bacteria is provided by the observation that the *S. citri dnaA* gene, which functions in DNA replication, is more distant from those of *S. apis* and *M. mycoides* than *dnaA* genes of walled bacteria are from one another (Suzuki et al., 1993). The observation suggests that divergence began in the walled bacteria much more recently than in the mollicutes. Longer branches on mollicute lineages than on lineages of other bacteria were also noted in a study of concatenated sets of more than 50 proteins present in all bacteria (Zhao et al., 2004), including phytoplasmas, but were interpreted to mean that evolution was more rapid in the mollicutes. The new concatenated protein study is consistent with the currently accepted view of *Mollicutes* evolution based primarily on 16S rDNA sequences (Woese et al., 1980). Relationships deduced from other genes, such as 5S rDNA (Walker, 1983), *dnaA* (Suzuki et al., 1993), and *pgk*, encoding phosphoglycerate kinase (Wolf et al., 2004), have provided supporting information.

The class is thought to have arisen by degenerative evolution from walled bacterial ancestors, as judged from nucleotide sequence (Woese et al., 1980) and lipid composition (Langworthy, 1983). In one scenario (Maniloff, 2002), birth of the class occurred around 605 million years ago (Mya), coincident with a major expansion in the diversity of marine animal life. About 470 Mya, about the time the first land plants appeared, the ancestral lineage split into two clades, one containing the *Acholeplasma*, *Aneroplasma* and phytoplasmas and the other composed of *Mycoplasma*, *Entomoplasma*, *Mesoplasma* and *Spiroplasma*. Phytoplasmas have to date not been cultured in vitro but are phytopathogens, indicating that plant pathogenicity has evolved at least twice among mollicutes. About 410 Mya, when the first land animals appeared and there was another major marine expansion, a split

occurred that led to the establishment of a major clade of *Mycoplasma*. The other branch led to the *Spiroplasma*, *Entomoplasma*, *Mesoplasma* and another clade of *Mycoplasma*, the mycoides cluster. A study of the progression of the numbers of lineages over time (Maniloff, 2002) led to the observation that *Mycoplasma* experienced a radiation into a variety of lineages about 191 Mya and that a similar radiation occurred for *Spiroplasma* about 100 Mya. Maniloff (2002) thought these events correspond well to the appearance of the first mammals and other vertebrates and to the radiation of flowering plants and their associated insects, respectively.

Phylogeny vs. Taxonomy

The phylogenetic patterns in these genera do not mirror the taxonomic groupings of organisms. The groupings had been based on a variety of phenotypic characters including absence of a cell wall, mol% G+C content of the DNA, genome size, sterol requirements, and other metabolic characteristics (Gasparich, 2002; Gasparich et al., 2004). The existence of two *Mycoplasma* clades means that this genus is not monophyletic. The pattern of *Spiroplasma* evolution is such that some lineages of this species had diverged before the cluster of *Entomoplasma* and *Mesoplasma* diverged from one of these lineages. As a result, this genus also is not monophyletic. A similar statement can be made for the *Entomoplasma* and *Mesoplasma* since the mycoides cluster diverged from a branch of this group of genera. As more and more sequences were obtained, more and more discrepancies between taxonomy and phylogeny became apparent.

The Big Picture

The picture to emerge from these phylogenetic considerations is that, if one were able to trace back the biogeographical niches of all the ancestors of any extant spiroplasma, one would find the lineage to be highly cosmopolitan, having spent time during its evolution in association with a variety of arthropod species, being deposited on or in a variety of higher plants and even occasionally becoming associated with vertebrate species. Often when an organism switches its host association, it initially causes disease in the new host. After a period of mutual adaptation, the pathogenicity is lessened until the relationship is one of commensality if not of mutualism. Thus phytopathogenicity may have arisen and disappeared multiple times during the evolution of spiroplasmas. However, as mentioned in the Introduction above, currently, only three phytopathogenic species are known.

Habitat

Plant Host Range and Specificity

Phytopathogenic spiroplasmas colonize the host plant's phloem sieve tubes, the tissue responsible for translocation of photosynthates from chlorophyllous photosynthetic tissues to metabolic sinks such as flowers, fruits, newly expanding leaves and storage organs. Although they are demanding in their growth requirements (and therefore dubbed "fastidious prokaryotes") spiroplasmas also colonize a second habitat that is very different from plant phloem—the hemolymph of insects in the Homoptera that transmit them from plant to plant (see the section Insect Transmission below in this Chapter). Although phloem sieve tubes and insect hemolymph share certain important features, both being rich in nutrients and serving as the transport for delivery of these compounds to other tissues, it is reasonable to hypothesize that colonization of these two ecological niches require different adaptations. Strong current interest in identifying genes differentially expressed in different environments will certainly contribute to our understanding of these microbes' ability to colonize different host tissues and to adapt rapidly as they shuttle between their plant and insect hosts.

CORN STUNT. The first reported observation of corn stunt was in 1942, when it was described in California's San Joaquin Valley (Stoner and Ullstrup, 1964). Three years later, the disease was diagnosed in South Texas, and reports followed from Mexico, Central and South America, all the states of the United States bordering Mexico, countries of the Caribbean Sea, and several Corn Belt states. The corn stunt spiroplasma also infects *Zea mays mexicana* and *Z. diploperennis*, *Z. perennis*, *Z. mays* X *Tripsacum floridanum*, and *Z. luxurians* (Nault, 1980). Experimental plant hosts include a range of monocotyledonous and dicotyledonous species; leafhoppers whose normal host range includes these species and that have been injected with the corn stunt spiroplasma can be used as a vehicle of infection.

The term "corn stunt" was used early on to describe a series of syndromes that later became distinguishable as different diseases caused by different pathogens or combinations of pathogens including *S. kunkelii*, the maize bushy stunt phytoplasma, and at least two viruses, maize chlorotic dwarf virus and maize rayado fino virus (Nault and Bradfute, 1979). Symptoms and impacts of *S. kunkelii* infection are described in the section Symptoms.

CITRUS STUBBORN. Citrus stubborn disease was first reported in California navel orange trees in 1915. The name "stubborn" was coined by citrus growers whose best efforts to cure their

trees were unsuccessful (Calavan and Oldfield, 1979). In 1928, the disease was discovered in several Mediterranean countries (being designated "little leaf" in Israel). It is now known to occur in most hot, dry citrus-growing areas around the globe but does not appear to be a serious problem in cool or humid areas. In contrast to *S. kunkelii*, *S. citri* (the citrus stubborn agent) is a generalist with a relatively broad host range. All citrus varieties grown in California are susceptible, but grapefruits, tangelos, and navel and Valencia oranges are particularly affected. Calavan and Oldfield (1979) identified natural infections in *Brassica tournefortii,* London rocket, pansy, rutabaga, turnip, wild radish, and wooly plantain. Later studies implicated other natural hosts and a large number of artificial ones. Although most are dicotyledonous, the pathogen also infects a few monocots, including onion. Symptomatology is described in the section Symptoms.

HORSERADISH BRITTLE ROOT. Although an unexplained disease of the southern Illinois specialty crop, horseradish (*Armoracea rusticana*), had baffled producers for a number of years, the sporadic nature of serious epidemics had hampered investigations into its cause. In 1984, a serious epidemic prompted investigation into the causal agent. Initially thought to be of viral etiology, the disease was found to be caused instead by the mollicute *S. citri* (Fletcher et al., 1981; Raju et al., 1981b). In epidemic years, some fields can be complete losses. Although *S. citri* already was known to infect a significant number of Brassicaceous species in California, the 1984 outbreak in Illinois horseradish was remarkable in its severe impact on the crop and its geographical distance from other known *S. citri* locations (Fletcher et al., 1983). Two years later, the same disease was found in horseradish plantings in Maryland (Davis and Fletcher, 1983). In Illinois, the spiroplasma often occurred in horseradish in mixed infections with viruses (Fletcher et al., 1984a) or with a phytoplasma (Eastman et al., 1984). Symptomatology is described in the section Symptoms.

Insect Host Range and Specificity

Although spiroplasmas have been isolated from most insect orders examined to date (Gasparich et al., 2004), transmission of plant pathogenic spiroplasmas has thus far been limited to leafhoppers (Order Hemiptera: suborder Homoptera: family Cicadellidae). Leafhopper vectors of spiroplasmas fall into a single taxon, the subfamily Deltocephalinae. Deltocephaline leafhoppers account for the majority of vectors of all leafhopper-transmitted pathogens, including leafhopper-borne viruses and phytoplasmas. This group of insects has been particularly well studied and has vector species in several tribes that make up the Deltocephalinae. The competent vectors of *S. kunkelii* include macrosteline leafhoppers *Dalbulus maidis* (Fig. 4) and *D. elimatus*. Other members of the genus are less efficient or nonvectors. Experimental vectors of *S. kunkelii* include *Cicadulina mbila* (the vector of maize streak virus), *Macrosteles sexnotatus*, *Graminella nigrifrons* (the vector of several maize viruses), *Stirellus bicolor*, *Euscelidius variegatus* and *Exitianus exitiosus*. The likelihood that any of these experimental vectors could be competent field vectors of *S. kunkelii* is low, with the possible exception of *C. mbila*. This species, a serious pest of maize and the primary maize streak virus vector, transmits the spiroplasma at 60% efficiency when given access to infected maize plants (Markham and Alivizatos, 1983b). Introduction of *S. kunkelii* into regions of Africa where *C. mbila*-maize systems are prominent could facilitate establishment of *S. kunkelii* on the African continent.

Fig. 4.   Two leafhopper vectors of phytopathogenic spiroplasmas: Left: *Dalbulus maidis*, vector of *Spiroplasma kunkelii*. Right: *Circulifer tenellus*, vector of *S. citri*. Photos: A. Wayadande (Panel A); M. Shaw and J. Fletcher (Panel B).

Table 1. Natural and experimental leafhopper vectors of the plant pathogenic spiroplasmas, *Spiroplasma citri* and *S. kunkelii*.

Vectors	References
S. citri natural/competent vectors	
Circulifer tenellus	Oldfield et al., 1976
	Eastman et al., 1988
Circulifer hematoceps	Fos et al., 1986
Circulifer opacipennis	Kersting and Sengonca, 1992
Scaphytopius nitridus	Kaloostian et al., 1975
	Oldfield et al., 1977
Scaphytopius acutus delongi	Kaloostian et al., 1979
S. citri experimental vectors	
Euscelidius variegatus	Markham, 1983
Euscelis plejebus	Markham and Townsend, 1974
Macrosteles fascifrons	O'Hayer et al., 1983
S. kunkelii natural/competent vectors	
Dalbulus maidis	Kunkel, 1946
Dalbulus elimatus	Nault, 1980
Dalbulus guevarai	Ramirez et al., 1975
S. kunkelii experimental vectors	
Euscelidius variegatus	Markham, 1977
Graminella nigrifrons	Nault, 1980
Exitianus exitiosus	Nault, 1980
Stirellus bicolor	Nault, 1980
Macrosteles sexnotatus	Markham and Alivizatos, 1983
Cicadulina mbila	Markham and Alivizatos, 1983

Leafhopper vectors of *S. citri* include *Circulifer tenellus* (Fig. 4), *C. haematoceps* (synonyms: *Neoaliturus tenellus* and *N. haematoceps*), *Scaphytopius nitridus* and *S. acutus delongi*. The host range of *S. citri* roughly coincides with the geographical distribution of *Circulifer* leafhoppers to include North America, the Mediterranean region, Africa, and Asia (Klein, 1992).

The degree of transmission specificity for both *S. citri* and *S. kunkelii* is high among the relatively small group of vectors for each pathogen (Table 1). This observed specificity of vector competency is primarily determined by vector host plant range. The *Circulifer* leafhoppers are polyphagous, feeding and reproducing primarily on dicotyledonous plants, including cruciferous hosts of *S. citri* (Cook, 1967). Interestingly, the economically important *S. citri* plant host, citrus, is not a preferred feeding and reproductive host of *Circulifer* leafhoppers, acquisition likely occurring from alternate weed hosts (Oldfield, 1988). The *S. kunkelii* vectors within the genus *Dalbulus* are specialists of either *Zea* spp. or *Tripsacum* spp. (Nault, 1990). *Dalbulus* leafhoppers are unlikely to feed on *S. citri* host plants, although the ability to feed and survive on phytoplasma-infected periwinkle was documented for *D. maidis* (Purcell, 1988). Similarly, the *S. kunkelii* plant host, maize, is unlikely to attract and support *Circulifer* spp.

Isolation and Cultivation

Cultivation

Spiroplasmas vary considerably in their adaptability to artificial media. In 1971, *S. citri* became the first spiroplasma, and the first phytopathogenic mollicute, to be cultivated in vitro when two research groups independently reported success using relatively simple media adapted from mycoplasma cultivation (Saglio et al., 1971; Fudl-Allah et al., 1972). The subsequent cultivation of *S. kunkelii* proved to be more challenging, however, because *S. kunkelii* failed to grow in media that supported rapid growth of *S. citri*, suggesting that the growth requirements of these two close relatives were different. By 1975, however, Chen and Liao (1975) succeeded in cultivating *S. kunkelii* using a relatively simple medium, while Williamson and Whitcomb (1975) achieved *S. kunkelii* cultivation by adapting formulations used for cultivation of *Drosophila* tissue cultures. Most spiroplasma media are relatively complex, chemically undefined mixtures (Chen and Davis, 1979; Lee et al., 2001). Animal sera are frequently used as a source of required cholesterol, an essential component of spiroplasma membranes, and undefined components such as yeast extract also are common. Also included are organic acids, amino acids, fatty acids, sugars,

inorganic salts and buffers. Recipes for a number of spiroplasma media are provided by Chen and Davis (1979), Liao and Chen (1982), Bové et al. (1983), Whitcomb (1983), and Lee et al. (2001).

Since the absence of a cell wall renders spiroplasmas vulnerable to osmotic lysis, successful ultivation has required the optimization of the osmotic pressure of culture media. Initial attempts were made to re-create the osmotic pressure within a sieve tube element, the phytopathogenic spiroplasmas' natural environment. Saglio et al. (1972) added both sucrose and sorbitol to their broth medium; the former as a carbon source and the latter to maintain an osmotic pressure of about 15 atm. The specific nature of the osmoticum may be important. High concentrations of sorbitol proved to be inhibitory for the growth of *S. kunkelii,* which grew much better at sorbitol concentrations about a third of those optimal for *S. citri* growth (Chen and Davis, 1979).

Temperature, pH and oxygen concentration are important factors in the growth of spiroplasmas. Most plant-associated spiroplasmas grow best at 30–32°C (Konai et al., 1992), their growth rates falling dramatically at warmer or cooler temperatures, while spiroplasmas from mammals can tolerate much higher temperatures (Chen and Davis, 1979). The optimal pH for most common spiroplasma media is 7.0–7.8. Higher or lower pH levels not only change the spiroplasmas' growth rate but also the highest titer reached and even the cell morphology (Chen and Davis, 1979). Changes in cell shape occur as a spiroplasma culture ages and acidic byproducts of metabolism accumulate, lowering the pH. While *S. citri* cultures are normally isolated and maintained in aerobic conditions, the corn stunt agent was isolated at higher frequency, and achieved a higher titer, when incubated in an anaerobic atmosphere with 5% CO_2 (Davis et al., 1984).

Isolation from Host Tissues

Initial isolation of spiroplasmas from infected plant tissue into artificial media presents challenges (Liao and Chen, 1982; Bové et al., 1983; Lee et al., 2001). Contamination by epiphytic microorganisms, whose growth rates are likely to far exceed those of the "fastidious" spiroplasmas, may be reduced by surface sterilization or by removal of the plant epidermis prior to sampling from the interior region. Samples are placed into a small volume (3–5 ml) of buffer or medium of suitable osmolarity and minced to increase the surface area from which spiroplasmas exude. During a short (5–10 min) incubation period at room temperature, spiroplasmas respond chemotactically, swimming out of the tissue into the sugar-rich medium. The medium-plus-

spiroplasma suspension is filtered (0.45 μm) to eliminate walled bacteria, fungal spores and plant debris. Because extracts from many plant species or tissues contain substances that inhibit spiroplasma growth (Liao and Chen, 1980; Liao and Chen, 1982; Bové et al., 1983), an immediate subculture (1:10–1:100) is commonly made to dilute the infected plant extract. Initial isolations are often done using media of high complexity and richness, as the spiroplasmas must adapt from the very rich environment provided in plant sieve tubes. Later on, spiroplasmas may be "weaned" from such requirements by gradually simplifying the medium (Whitcomb, 1983). Culture establishment can be monitored conveniently by 1) the development of light turbidity in the culture tubes (dense turbidity usually indicates contamination by more prolific species, and because spiroplasmas are both motile and small enough to exhibit Brownian motion, the presence of a pellet at the bottom of the tube also indicates contamination); 2) a color change in an acid-base indicator, indicating the presence of acidic byproducts of metabolism; or 3) an increase in spiroplasma cell counts. The latter are often evaluated using light microscopy and dark-field optics (Tully, 1983) at a magnification of 1000–1250X, at which the helical morphology of spiroplasmas is easily apparent. Helices can also be viewed, but far less clearly, using phase contrast optics at the same magnifications (Bredt, 1983).

Plant pathogenic spiroplasmas also may be isolated from the bodies of their insect vectors, a procedure that, like isolation from plant tissue, may be challenging because of low pathogen titer and the presence of growth inhibitors in the host tissue. Although many species of insect-associated spiroplasmas colonize only the gut lumen of their insect hosts (Markham et al., 1983c), all of the plant pathogenic species invade the body cavity by traversal of the gut epithelium and multiply to relatively high titers in the hemolymph. In some cases the bacterial population reaches higher levels in the hemocoel than in plant phloem, making isolation from insects an attractive alternative (Markham and Townsend, 1979).

As spiroplasma cultures mature, the growth conditions become less optimal; certain nutrients begin to become limiting, the pH of the medium falls, and toxic byproducts may reach concentrations detrimental to the cells. The spiroplasmas divide less frequently under such conditions, and as a result they become longer and have more turns to the helix, but the amplitude of the helical turns decreases as the helices relax. Whereas in young cultures cell division is followed quickly by separation of the daughter cells, older cultures contain many aggregates of spiroplasma cells (called "Medusa's heads" because the helical

cells radiating from a central point resemble the tresses of the famous mythological figure) that presumably result from a failure of daughter cells to separate. As cells age further, cell membrane changes occur, and "blebs" or bulges become visible along the length of the filaments. Finally, the cells lose the filamentous form altogether, assuming a globular shape. In this globular stage the spiroplasmas are generally unrevivable, but at previous stages, the short, tight helical morphology and rapid cell division characteristic of a healthy population can be restored by transferring a small volume into fresh medium (see section Morphology in this Chapter).

Chemically undefined media continue to support the most rapid and prolific growth of many spiroplasmas, but such media are suboptimal for investigations of nutritional requirements of spiroplasmas. In 1982 the first chemically defined spiroplasma medium, CC-494, was reported for some epiphytic species (Chang and Chen, 1982). Although microbial growth rates and maximum titers were reduced, the medium facilitated nutritional analyses. A defined medium for *S. citri* was developed soon after (Lee and Davis, 1983). Lee and Davis (1984) reported that the growth of *S. citri* in LD8 broth reached a titer of over 6×10^9 colony forming units (cfu)/ml at a doubling time of 4 h at 31°C, while *S. kunkelii* reached 2×10^9 cfu/ml, doubling every 11–12 h, at 31°C in LD8A.

Cultivation on Solid Media

All three of the plant pathogenic species of *Spiroplasma* produce colonies on agar-solidified media. Colony sizes vary depending on the titer of the plated suspension, the nutrient content of the medium, and the concentration of the agar. As discussed in the section Morphology, colonies of normally motile spiroplasmas are small, and their edges are indistinct, appearing to be "out of focus," because of the formation of numerous satellite colonies by the swimming cells (Fig. 3).

Colonies of certain nonhelical strains, such as the naturally occurring *S. citri* strain ASP-1, more closely resemble the familiar "fried egg" colonies of zoopathogenic and human mycoplasmas.

When establishing research cultures from a natural source, it is advisable to establish a homogeneous spiroplasma population by subjecting the primary culture to a triple filter-cloning step, using a 0.22-μm filter to remove spiroplasma aggregates and selecting single colonies for propagation (Lee et al., 2001).

Identification and Detection

Many methods are available, and in use, for the detection and identification of phytopathogenic spiroplasmas. The choice of a particular test or tests will depend on the degree of certainty and discrimination required, the specific information desired, and the capabilities of the laboratory in which testing is done.

Dienes' Stain

For rapid assessment of plant samples from field collections, a quick and easy preliminary test for either spiroplasmas or phytoplasmas is to apply Dienes' stain to hand-cut sections of tissue (Deely et al., 1979). Originally developed to aid in visualization of mycoplasma colonies on agar surfaces, the stain directly colors the pathogens so that phloem sieve tubes colonized by spiroplasmas contain irregular patches of deeply stained cells when viewed by light microscopy (Fig. 5). Dienes' stain has some limitations, however. To avoid false positives, it is advisable to include known negative samples in the analysis, and Dienes' staining may be difficult to interpret in older or woody tissues. In addition, Dienes' positive staining reactions have been reported for diseases caused by non-mollicute phloem-resident plant pathogens such as *Serratia marcescens*, causal agent of cucurbit yellow vine

Fig. 5. Horseradish root cross section, stained with Dienes' stain. Large, thick-walled xylem vessels of both healthy (Panel A) and diseased (Panel B) plants retain the blue dye. Blue-stained cell patches (arrow) occur in the phloem region in diseased plants only. Photos: J. Fletcher.
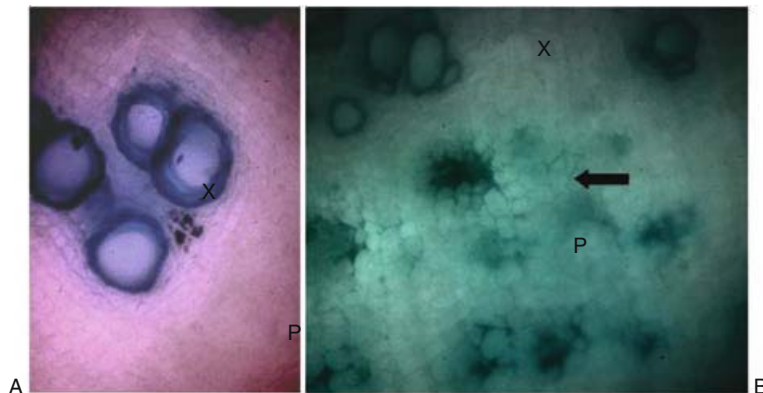
disease, and beet curly top virus in pepper, sugar beet and bean (Bruton et al., 1998).

DAPI

The fluorescent dye DAPI (4′,6-diamidino-2-phenylindole) binds to DNA in a nonspecific manner. Although the binding is nonspecific, labeling host DNA in the nucleus and organelles as well, the dye has been used widely for detection of mollicutes within plant tissues (Seemuller and Kirkpatrick, 1996; Lee et al., 2001).

Electron Microscopy

Because it is tedious and expensive, and because phytopathogenic mollicutes are often distributed irregularly within a plant host and thus may be missed, pathogen visualization at the electron microscope level is used more commonly for the uncultivable phytoplasmas than for spiroplasmas. However, electron microcroscopy (EM) is still useful for spiroplasma detection and has the advantage of showing the wall-less cell morphology characteristic of the mollicutes (Williamson, 1983). Although the long and helical spiroplasmas are often cut in cross- or transverse sections when typical thin sections of tissue are prepared, making spiroplasmas difficult to distinguish from phytoplasmas, "thick" thin sections can be cut that allow visualization of the helical turns of spiroplasmas. Specific considerations for optimal sample collection and preparation, and viewing of spiroplasmas via EM, are available (Waters, 1982; Cole, 1983; Carson and Collier, 1983; Garnier and Bové, 1983; Williamson, 1983; Chen et al., 1989).

Isolation and Cultivation

Detection of phytopathogenic spiroplasmas in infected plants is often accomplished by cultivation in artificial media (see section Cultivation in this Chapter). Because contamination with more aggressive, walled bacteria is a concern, culture media may be supplemented with antibiotics such as tetracycline, which affect protein synthesis rather than cell wall formation, and are generally viewed using light microscopy and dark field optics for high-contrast visualization of typical helices in the media. Isolation can be quantitative if cultivation is done on agar-solidified media, and if samples are adequately replicated and controlled (Kloepper et al., 1982).

Serology

Various strategies in which antibodies, either polyclonal or monoclonal, are used to differenti-ate spiroplasma taxa, have been developed. The growth inhibition test (Taylor-Robinson, 1979), which can discriminate among major spiroplasma serogroups, is performed by placing filter paper discs saturated with specific antiserum onto lawns seeded with spiroplasmas and measuring the resulting zones of growth inhibition. The metabolism inhibition test is similar in concept but carried out in broth medium supplemented with an acid-base indicator dye; the assay is evaluated by a color change. The organism deformation test (Williamson et al., 1978), commonly used to place a spiroplasma into a subgroup, is based on the ability of a specific antibody to alter the spiroplasma's outer membrane surface, resulting in the loss of the helical morphology. Affected cells take on a spherical shape that is readily seen in dark field microscopy. Quantitative relationships among various spiroplasmas may be determined by titration of the antisera. The metabolism inhibition and organism deformation tests may be combined, as reported by Williamson et al. (1979). Anti-spiroplasma antibodies can also be used in simpler, faster assays such as latex agglutination (Fletcher and Slack, 1986) and dot-immunoblotting (Fletcher, 1987).

In the early 1980s, enzyme-linked immunosorbent assay (ELISA) was adapted for the detection and identification of phytopathogenic spiroplasmas. Raju and Nyland (1981a) and Eden-Green (1982) both used the test for detection of the corn stunt spiroplasma in infected plants or extracts therefrom, at concentrations of 10^6 cells/ml or higher. In the latter study, false positives were a problem when leafhoppers (*Dalbulus maidis*) were tested. ELISA also detected up to 10^9 *S. citri* cells per gram of *Catharanthus roseus* (then *Vinca rosea*) tissue and detected the pathogen in samples as small as a single leafhopper (Archer et al., 1982). Lin and Chen (1985) were the first to report the preparation of anti-*S. citri* monoclonal antibodies and their use to discriminate among strains of the spiroplasma. Interestingly, in 1996 the failure of extracts of symptomatic periwinkle plants (collected from *S. citri*-affected citrus groves in the Middle East) to react in ELISA with antiserum against *S. citri* demonstrated the existence of a third species of plant pathogenic spiroplasma that was later designated "*S. phoeniceum*" (Saillard et al., 1996). As with cultivation, ELISA also may be quantitated for use in determining population levels in plant hosts, in different tissues, and over ranges of time (Saillard et al., 1978; Daniels et al., 1982; Fletcher and Eastman, 1984a; Gussie et al., 1995). ELISA test kits, as well as individual antibodies and control samples (positive and negative), for both *S. citri* and *S. kunkelii* are available commercially.

Polyacrylamide Gel Electrophoresis

Before the advent of convenient molecular fingerprinting techniques, polyacrylamide gel electrophoresis (PAGE) patterns in both one and two dimensions were used widely to compare and discriminate among serogroups, subgroups, and strains of plant pathogenic spiroplasmas (Davis and Lee, 1982; Fletcher et al., 1995). In addition, PAGE analyses allowed the clustering of strains into distinct groups, each containing highly related strains. Such analyses later proved to agree well with other methods of identification and classification.

DNA-DNA Hybridization and DNA Probes

As the number of spiroplasma strains increased (most being isolated from arthropod species), the need to more accurately assess their relationships and phylogeny increased. Early comparisons of spiroplasma genome sequences by DNA-DNA homology (Christiansen et al., 1979; Junca et al., 1980; Lee and Davis, 1980) showed that taxonomies and phylogenetic histories suggested by such analyses were in good agreement with the serological data accumulated previously, and taxonomic groupings of spiroplasmas began to be developed. DNA-DNA hybridization has gradually been replaced by the less-difficult amplification and analysis of 16S rDNA sequences.

Polymerase Chain Reaction

Use of the conserved sequences of 16S rRNA have proved to be at least as valuable for the detection of wall-less prokaryotes as for their walled relatives (Deng et al., 1992; Saillard et al., 1996), and even more valuable for the uncultivable phytoplasmas. The method is particularly useful for detection of the mollicutes within their host tissues, plant phloem, or insect vectors, because primer specificity allows the pathogen DNA to be preferentially amplified. Using primers designed from spiralin or from spiroplasma virus DNA sequences, Saillard et al. (1996) developed polymerase chain reaction (PCR) for detection of *S. citri* in periwinkle plants, achieving sensitivity from 100–1000 times greater than that of ELISA. Other common primers are developed from the 16S rRNA genes or from other genes that are highly conserved among taxa. In some cases host components may interfere with the amplification, or spiroplasma titers may be very low in some tissues, giving rise to false negative reactions. Optimization protocols include dilution of the host inhibitors or partial sample purification steps. Altered versions of

PCR also exist. In nested PCR, two primer pairs (one "universal" set and the other either universal or species-specific) are used in tandem to amplify mollicute DNA from very low populations. Immunocapture PCR enriches for spiroplasmas using specific antibodies, giving 10X greater sensitivity in subsequent PCR reactions (Saillard et al., 1996). Bastion and Foster (2001) recently reported identification of a single primer pair that amplified a 276-bp fragment from all spiroplasmas tested except for MQ1 but did not amplify other mollicutes tested. Such primers might be useful as "universal" for spiroplasma detection. On the other hand, primers unique for specific plant pathogenic spiroplasmas also have their place; Barros et al. (2001) recently developed primers based on *S. kunkelii*-unique regions of the sequence of the spiralin gene for detection of the corn stunt spiroplasma but not of *S. citri*.

Genomic Sequence

When the complete genomic sequence is available for the two most commonly occurring phytopathogenic spiroplasmas, *S. citri* and *S. kunkelii* (see the section DNA Sequence in this Chapter), researchers will have the ability to identify any gene or nucleic acid sequence that would differentiate between these two species. Having the genomic sequences also will assist in the selection of genus-specific regions that could be useful in disease diagnosis and pathogen identification. A simple application would be to design PCR primers for amplification of a sequence or sequences previously determined to be discriminatory. Detection of a PCR product may be followed by sequencing of the product to distinguish further among spiroplasmas. See also the section Phylogeny and Taxonomy in this Chapter.

Pathogenicity Tests and Host Range

Because they colonize the phloem, spiroplasmas can be transmitted from plant to plant in some host species by grafting or via the parasitic plant, dodder (*Cuscuta compestris* or *C. subinclusa*; Lee et al., 2001). Madagascar periwinkle, *Catharanthus roseus*, is a commonly used host for the maintenance and propagation of plant pathogenic mollicutes, including spiroplasmas, because it is readily available, easy to grow, readily grafted, and susceptible to most of the mollicute pathogens. Furthermore, the symptoms produced on periwinkle vary with the pathogen species, significantly in some cases and subtly in others; seasoned researchers are able to distinguish such symptoms.

Preservation

Storage of spiroplasmas may be more challenging than for other bacteria because of the absence of a cell wall. Mid- to late-log phase cultures quick-frozen in small aliquots (100–500 μl) of liquid growth medium at ultra-low temperature (–70° to –80°) or in liquid nitrogen generally remain viable for a number of years; freezing at warmer temperatures may be successful for shorter periods. Lyophilization (freeze-drying) in growth medium is preferred for extended storage (Leach, 1983). A quick and easy method for transfer or mailing of spiroplasmas is to place drops of log-phase cultures on small discs of filter paper and dry them in a desiccator without vacuum; such cultures can be revived after 6 wk at room temperature and 3 months at 4°C (Liao and Chen, 1982). Because spiroplasmas cannot be mechanically transmitted to plants, a disadvantage of this method of storage is that plant inoculations require insect vector acquisition, either by feeding the insects on spiroplasma suspensions or by needle inoculation into the hemocoel of the insect vectors. Because of the high natural mutation rate of spiroplasmas in culture (see section Genetics in this Chapter), storage of the early passage cultures of plant or insect isolates is very important. Aliquots of both the original (uncloned) isolate as well as of several clones of the triply cloned population should be stored.

For convenience in plant experiments, plant pathogenic spiroplasmas are often maintained in plant hosts or insects or both. Long lived plant species that are easy to grow and amenable to grafting, such as Madagascar periwinkle (*Catharanthus roseus*), are usually chosen to circumvent the labor-intensive requirement for insect inoculations. However, long-term lack of exposure of the spiroplasmas to their insect vectors (and the accompanying lack of selection pressure from that part of their normal lifecycle) may lead to loss of the ability to be insect transmitted. For example, a strain of *S. citri* BR3-3X (maintained for 10 years in periwinkle by successive grafting) was found to be nontransmissible by the beet leafhopper, *C. tenellus* (Wayadande and Fletcher, 1995). Several researchers have noted also that spiroplasma strains passaged by grafting may lose virulence for the plant host over time. After several years of in planta graft maintenance, *S. citri* strains once able to kill a periwinkle host plant in 1–2 weeks produce only a mild mottling, and infected host plants may survive for months.

Physiology and Metabolism

The physiology of members of the Class Mollicutes has been studied primarily in the genus *Mycoplasma*; for excellent reviews, see Pollack (1992), Finch and Mitchell (1992), Fischer et al. (1992), McElhaney (1992), and Pollack et al. (1997). The first spiroplasmas to be discovered, plant pathogens, were designated "fastidious" prokaryotes because of the difficulty encountered in their cultivation and the complex mixtures of compounds ultimately required for their in vitro growth (see the section Cultivation in this Chapter). Interestingly, phytoplasmas, the other plant pathogens of the Class Mollicutes, remain uncultivable despite focused efforts to identify suitable media. The resistance of these plant pathogenic mollicutes to cultivation probably reflects the loss of metabolic functions resulting from adaptation to a parasitic lifestyle in their plant hosts and insect vectors.

Intermediary Metabolism (Sugars and Energy)

RESPIRATORY PATHWAYS. The spiroplasmas are unusual in their energy-generating mechanisms. Early investigators (Hollander et al., 1977) suggested that respiratory chains were absent and the microbes' inability to reduce redox indicators support that conclusion (Pollack et al., 1996). Unlike other spiroplasmas, however, the phytopathogenic spiroplasmas do have oxidoreduction sites, but they occur exclusively at the blunt end of the organism (Garnier et al., 1984). Organisms having respiratory chains use the generated energy to synthesize ATP via a proton-ATPase. A proton-ATPase has been identified in spiroplasma by serology (Rottem et al., 1987), but since the microbes lack the other components of respiratory metabolism this enzyme likely functions in pH stasis.

Lacking oxidative metabolism, phytopathogenic spiroplasmas use the nonoxidative breakdown of sugars to generate energy. The effectiveness of the pathway is shown by the fact that the adenylate energy-charge of tested mollicutes is similar to that of *S. citri* and other bacteria (Beaman and Pollack, 1983). Genes for each of the enzymes of the glycolytic pathway can be recognized in the genome sequence of *S. kunkelii* (Table 2). Although in most organisms the enzyme lactate dehydrogenase is very specific for its substrate, in some mycoplasmas it also functions as a malate dehydrogenase. In *S. melliferum*, a close relative of *S. citri,* there are two separate enzymes (Cordwell et al., 1997). Curiously, no evidence of a gene for a separate malate dehydrogenase is found in the *S. kunkelii*

Table 2. Distribution of functions of genes identifiable in the *S. kunkelii* genome project.

Class	Number	Percent
?	1	0.1
Biosynthesis of cofactors, prosthetic groups, and carriers	6	0.5
Cell envelope	26	2.3
Cell/organism defense	2	0.2
Cellular processes	20	1.7
Central intermediary metabolism	11	1.0
DNA metabolism	40	3.5
Energy metabolism	21	1.8
Fatty acid and phospholipid metabolism	4	0.3
Hypothetical proteins	96	8.3
Protein fate	6	0.5
Protein synthesis	60	5.2
Purines, pyrimidines, nucleosides, and nucleotides	16	1.4
Regulatory functions	1	0.1
Transcription	8	0.7
Transport and binding proteins	38	3.3
Unknown	678	58.8
Viral	120	10.4
Total	1154	100.0

genome sequences (Table 2). The ability to oxidoreductively exchange malate and oxaloacetate undoubtedly plays a role in the maintenance of oxidized to reduced free nicotinamide-adenine dinucleotide (NAD+/NADH) ratios.

SUGAR METABOLISM. The sugars available to phytopathogenic spiroplasmas are different in their two eukaryotic hosts. In plants, where spiroplasmas colonize phloem elements, they are exposed to relatively high concentrations of sucrose and to lower concentrations of its component monosaccharides, glucose and fructose. In contrast, the disaccharide trehalose is the major sugar in leafhopper hemolymph (Andre et al., 2003), but this sugar is composed only of glucose. Thus, one can expect that the spiroplasmas have a versatile sugar metabolism that can adapt to two substantially different environments, one glucose-rich and the other a balanced glucose-fructose environment.

The ability of the spiroplasmas to grow using trehalose apparently stems from an ability to import and hydrolyze the sugar. The genomes of *S. citri* (W. Maccheroni and J. Renaudin, cited in Bové et al., 2003) and *S. kunkelii* contain a three-cistron operon (U. Melcher, unpublished observations; see also http://www.genome.ou.edu/spiro.html {Spiroplasma kunkelii Strain CR2-3x Genome Sequencing Web site}). The second cistron encodes a trehalose permease component, IIBC, of a phosphotransferase system, while the third encodes the amylase needed to hydrolyze the disaccharide. The other component of treha-

lose permease II contains a IIA domain like those that, in most other sugar permeases, are covalently attached to IIBC domains. The IIA-domain polypeptide is synthesized from a separate gene (Andre et al., 2003). It also serves as the IIA component of a glucose permease, whose IIBC component is encoded by still another gene. The first cistron of the trehalose operon, *treR*, putatively encodes a positive regulator of the trehalose operon.

In contrast, in plants, the disaccharide is not taken up by the spiroplasma and subsequently hydrolyzed. Indeed, the spiroplasmas are unable to grow on sucrose as a carbon source. However, they are able to take up and metabolize glucose and fructose by separate pathways. Glucose uptake is mediated by a glucose permease, whereas fructose is taken up and phosphorylated by a fructose permease, the product of the *fruA* gene. The fructose-1-phosphate is further phosphorylated to fructose-1,6-bisphosphate by 1-phosphofructokinase, the product of the *fruK* gene. The phosphofructokinase itself is probably not strongly regulated by phosphoenolpyruvate as in many other bacteria, since it has an aspartic acid at position 187 (Auzat et al., 1994); only tightly regulated enzymes have a glutamic acid at this position. The *fruA* and *fruK* genes form an operon analogous to the trehalose operon, with a fructose regulatory cistron, *fruR,* located as first cistron of the *fru* operon. The *fru* operon is regulated by fructose through FruR (Gaurivaud et al., 2001), which likely is an activator of transcription.

Inactivation of the *S. citri* fructose utilization gene cluster (Gaurivaud et al., 2000) by insertion mutagenesis or selection for xylitol resistance (Gaurivaud et al., 2000) gave rise to spiroplasmas that produced delayed and milder symptoms on periwinkle plants (Gaurivaud et al., 2000). Complementation of mutants with fructose operon genes restored both fructose utilization and wildtype pathogenicity. Fructose in culture stimulates transcription of the operon only when an active *fruR* is present, supporting the identification of FruR as a positive transcription regulator. Fructose utilization may inhibit phloem loading of sucrose in the source leaves. The resulting deficiency of sugar in sink leaves and excess in the source leaves may lead to pathogenic effects (Bové and Garnier, 2002; Bové et al., 2003; see also the section Virulence Mechanisms in this Chapter). Still unclear is whether spiroplasmas are restricted to scavenging the low concentrations of glucose and fructose available in phloem or whether they actively alter plant metabolism to provide the bacteria greater fluxes of sugars. The overall picture to emerge is that appropriate positive regulation of the host-specific pathways ensures that only the

pathway appropriate for the host of residence is active at any one time.

Regulation

Regulation via *fruR* and, putatively, via *treR* represent two of but a handful of documented or suspected regulatory mechanisms in phytopathogenic spiroplasmas. *Spiroplasma citri* does not contain calmodulin, a protein that could modulate calcium-dependent regulation (Barth et al., 1991). Regulation by protein phosphorylation is a possibility since a protein of 57 kDa from *S. melliferum* is phosphorylated in vivo (Platt et al., 1990). *Spiroplasma citri* can obtain energy from arginine (Townsend, 1976), and both *S. citri* and *S. kunkelii* contain an aminopeptidase active on arginine substrates (Stevens et al., 1984). Growth of these spiroplasmas in the presence of arginine reduced the level of the enzyme accumulated, suggesting a feedback regulation. The pathway of ATP-generating arginine catabolism in mollicutes requires the enzymes arginine deiminase, ornithine carbamoyltransferase, and carbamate kinase, for which encoding genes were detected in the *S. kunkelii* genome (U. Melcher, unpublished data).

Lipids and Membranes

The basic outlines of spiroplasma lipid metabolism were sketched by feeding studies and compositional analyses (Freeman et al., 1976). Phytopathogenic spiroplasmas cannot synthesize fatty acids and thus require exogenous fatty acids for growth (Pollock, 1987). Spiroplasmas take up phospholipids directly and incorporate them into membranes with little or no modification (Rottem et al., 1986). Membranes contain sphingomyelin (Razin et al., 1973; Davis et al., 1985) and cholesterol and are rich in phosphatidylglycerol (Mudd et al., 1977), of which spiroplasmas can synthesize only the latter. The *S. kunkelii* genome contains genes that likely produce an acyl carrier protein (ACP), an ACP phosphodiesterase, a CDP-diacylglycerol kinase, and glycerol-3-phosphate dehydrogenase, enzymes having key roles in phospholipid metabolism. *Spiroplasma citri* and other spiroplasmas, in contrast to mycoplasmas, have neither lipopolysaccharide-like (Smith et al., 1976) nor lipoglycan (Smith, 1984) molecules. The membranes of the closely related phytopathogens *S. citri* and *S. kunkelii* are likely to be very similar in composition and function, but although their compositions were judged identical in one study (Patel et al., 1978), they were found to have minor differences in another (Mudd et al., 1979).

Proteins

GENERAL. The protein content of spiroplasmas can be fractionated by electrophoresis and characterized (Cordwell et al., 1995; Cordwell et al., 1997). Some spiroplasma proteins separated by two-dimensional (2-D) gel electrophoresis (Wroblewski, 1981) lacked extensive disulfide crosslinkages. One of these, D16, is an 89-kDa protein (probably identical to spiroplasma adhesion-related protein (SARP)1, an adherence-associated protein of *S. citri*; Berg et al., 2001) that shows amino acid sequence similarity to an adhesin of *M. agalactiae* (Fleury et al., 2002; see the section Molecular Biology in this Chapter). Even 1-D gel electrophoresis is useful; protein profiles of *S. citri* lines differing in pathogenicity and transmissibility characteristics showed phenotype-related pattern differences including the presence of a 144–146 kDa band in transmissible lines and its absence in nontransmissible ones (Fletcher et al., 1996).

MEMBRANE PROTEINS. The membrane proteins of phytopathogenic spiroplasmas may be isolated by several means. For example, those of *S. citri* can be isolated by extraction with sodium desoxycholate (Wroblewski, 1975), sarkosyl (Wroblewski et al., 1978), or by alternate cycles of detergent (such as Triton-X 114) and aqueous extraction (Fletcher and Wijetunga, 1981).

Proteins having surface domains on the phytopathogen *S. citri* BR3 were identified by their sensitivity to proteases and antibodies and by ^{125}I-labeling studies (Fletcher et al., 1989). Most surface-exposed proteins of *S. citri* are resistant to proteinase K (Yu et al., 1997; Yu et al., 2000), but the adherence-associated protein SARP1 is readily cleaved, leaving behind a 48-kDa remnant. Cleavage correlated with loss of the spiroplasmas cells' ability to bind to leafhopper cells in culture. On the other hand, others (Butler et al., 1991) have found general susceptibility of spiroplasmas membrane proteins to proteinase K and identified a proteinase resistant protein, p40-pr, of unknown function.

As with other bacteria, spiroplasma membranes contain a number of enzymes (Kahane et al., 1977; Mudd et al., 1977). Using zymograms and zymoblots to identify enzyme functions in *S. citri,* Wagih and Fletcher demonstrated that *S. citri* possessed esterase, ribonuclease and tyrosinase activities but lacked several enzymes common to most prokaryotes (Wagih and Fletcher, 1988; Wagih and Fletcher, 1993). Several important enzymes were demonstrated to be present directly in osmotic lysates of spiroplasmas, including *S. citri* (Chen and Chang, 1994).

SPIRALIN. Spiralins (Bové et al., 1993) are abundant, acylated (Wroblewski et al., 1989)

proteins of about 29 kDa covering the spiroplasma plasma membrane like a carpet (Castano et al., 2002). Minor amounts of a larger spiralin form are present within *S. citri* cytoplasm, suggesting that a prespiralin is processed to remove a prepeptide (Mouches et al., 1984). When synthesized in *E. coli* (Mouches et al., 1985) from a transgene (Bové et al., 1984), spiralin was found in the cytoplasm and in both the inner and outer membranes, in three molecular weight forms (Blanchard et al., 1987). Spiralins are molecularly heterogeneous because of posttranslational modifications (Foissac et al., 1996), possibly variable acylation of the N-terminus and the *S*-glycerylcysteine (Wroblewski et al., 1987; Le Henaff and Fontenelle, 2000; Le Henaff and Fontenelle, 2002).

Probably as a result of the lipid moieties attached, spiralin is an intrinsic membrane protein (Wroblewski, 1979), resistant to agents removing peripheral proteins and extractable by detergents (Blanchard et al., 1985; Plusquellec et al., 1989). Of interest to spiralin's relationship to membranes is a 20-residue, putatively amphipathic peptide (Bondon et al., 1995). Circular dichroism studies show such spiralin peptides to have unordered structure in aqueous solution but to form alpha helices in lipid micelles (Brenner et al., 1995).

Spiralin sequences, deduced from their gene sequences from *S. melliferum* (Chevalier et al., 1990), *S. citri* (Chevalier et al., 1990), *S. phoeniceum* and *S. kunkelii* (Foissac et al., 1997), reveal evidence of positive selection for substitutions that alter the protein's amino acid sequence (Foissac et al., 1997). This observation, and the prevalence of the protein on the cell surface, support the hypothesis of a major role for spiralin in host interactions. This hypothesis was supported when disruption of the spiralin gene by homologous recombination using an *oriC*-derived plasmid resulted in a considerable decrease in leafhopper transmissibility despite no decrease in pathogenicity (Duret et al., 2003), indicating that the spiralin-relevant host interaction is with the leafhopper, rather than the plant host.

Nucleic Acids

The small cell size of spiroplasmas compared to other bacteria is accompanied by a small genome that lacks a considerable number of common bacterial genes (see the section Genetics: General Characteristics in this Chapter). Nevertheless, *S. kunkelii* exhibits a small repertoire of easily recognized genes for enzymes of purine and pyrimidine metabolism (U. Melcher, unpublished observations; Table 2): cytidine deaminase, uridine and thymidine kinases, cytidylate kinase, purine-nucleoside and thymidine phos-

phorylases, thymidylate synthase, and GMP reductase.

Genes involved in DNA replication and repair have been studied in both *S. citri* and *S. kunkelii*. The former spiroplasma contains at least two DNA polymerases (Charron et al., 1979) and probably three (Charron et al., 1982), as in *A. laidlawii* but not as in *M. mycoides*, which has but a single enzyme (Maurel et al., 1989). The *S. kunkelii* genome sequence shows clear evidence of the presence of genes for DNA polymerase I and III homologs but not for a DNA polymerase II. In addition, signatures of genes for other enzymes involved in DNA synthesis and function are present: DNA topoisomerases I, II and IV, DNA primase, DNA ligase, DNA helicase, and single-stranded DNA binding protein. Several DNA repair enzyme genes can also be recognized, those encoding: DNA photolyase, excinuclease ABC, endonuclease IV and formamidopyrimidine- and uracil-DNA glycosylases. Earlier studies demonstrated that *S. citri* is more sensitive to ultraviolet (UV) radiation than is *Escherichia coli*, but the UV sensitivity curves do indicate the presence of some DNA repair capabilities (Labarere and Barroso, 1984). No evidence for a dark repair system was obtained, but the presence of an SOS type repair system was suggested (Labarere and Barroso, 1989). The *recA* gene of *S. citri* is truncated and probably not functional; that of *S. melliferum* contains a stop codon in its middle (Marais et al., 1996), but the gene may be functional, as the stop codon is likely read-through (Melcher et al., 1999b).

Genetics

The general characteristics of spiroplasma genomes have been known for some time, as have a few nucleotide sequences, but much more has been learned in recent years. Sequence information has burgeoned and genetic mechanisms are being characterized. Extrachromosomal elements, consisting of plasmids and phage replicative forms (RF), are being better delineated. Methods for genetic characterization by gene introduction and gene inactivation have been developed and are yielding significant insights.

General Characteristics

G+C CONTENT AND METHYLATION. The plant pathogenic spiroplasmas have G+C contents of 25–27 mol%, as do several related spiroplasmas, levels even lower than the 29–32% found in other spiroplasmas (Bové et al., 1982). The sizes of spiroplasma genomes vary widely (Carle et al., 1995); those of the phytopathogenic

spiroplasmas are 1.5–2.2 Mbp but can change rapidly by mutation within even a few generations (Ye et al., 1996). Consistent with the small genome size, spiroplasmas, like several other mollicutes, contain a single rDNA set (Razin et al., 1984a; Razin et al., 1984b); mollicutes in general have a lower number of rRNA genes than their larger genomed relatives (Amikam et al., 1984). The states of DNA methylation are highly divergent among the spiroplasmas (Nur et al., 1985), and both cytosine and adenine can be targets of methylation. Although methylcytosine occurs only in CA and CT sequences in *S. apis*, in *S. floricola* it is found in all CN dinucleotides. The methylation occurs by modification methyllases, which protect the organism from their own restriction endonucleases. Spiroplasmas encode multiple restriction endonucleases. For example, *Sci*N1, which has been purified and characterized, cleaves at CGCG sequences and is thought to play a role in protecting *S. citri* against phage infection (Stephens, 1982).

REPETITIVE ELEMENTS. Short repetitive sequences are present in the chromosomes of *S. citri*, *S. kunkelii* and *S. phoeniceum* strains, but not in those of nine other spiroplasma strains examined (Nur et al., 1987). Similar repeats found in a cloned fragment of the abundant (Razin et al., 1987) plasmid pRA1 from *S. citri* R8A2 were derived from an SpV3 phage (Ranhand et al., 1987). On long passage in culture, the pRA1 sequences become integrated in the chromosome (Razin et al., 1987).

GENE ORDER. Comparison of a restriction map of *S. citri* R8AH2.HP, published in 1992 (Ye et al., 1992), with maps generated for *S. melliferum* (Ye et al., 1994) and other spiroplasmas revealed considerable heterogeneity among group I spiroplasmas, even within the *S. citri* species (Ye et al., 1995a; Ye et al., 1996). However, the finding that synteny between *S. citri* and *S. melliferum* is violated by only a single inversion strongly supports the recent evolutionary divergence of these two genera (see the section Phylogeny and Taxonomy in this Chapter).

Genetic Code

Spiroplasmas have an unusual nucleotide codon usage feature. They contain two tRNA$_{trp}$, one recognizing UGG and the other recognizing UGA, as first reported for *S. citri* by Citti et al. (1992). This observation and the finding that several protein coding genes contained in-frame UGA codons suggested that in spiroplasmas UGA is not a stop codon but a tryptophan (trp) codon. The fact that mutation of UGG to UGA did not stop expression of the gene in which the mutated UGG trp codon was located (Stamburski et al., 1992) provided further evidence that

UGA coded for trp in *S. citri*. The same codon usage is found in other spiroplasmas as well.

Protein Processing

Proteins synthesized by the translational machinery may be processed by a typical signal peptidase. That spiroplasmas have a signal peptidase gene was first inferred because the peptidase inhibitor globomycin prevented processing of spiralin, the major protein of the external surface (Beven et al., 1996). More recently, several other spiroplasma proteins, for example SARP1 (Berg et al., 2001), have been characterized as having a mature N-terminal amino acid sequence corresponding to expectation from signal peptidase processing.

DNA Sequence

Until recent technology permitted the sequencing of entire bacterial genomes, gene sequence analysis focused on particular genes of regions of interest. Early studies of *S. melliferum* included the characterization (Rogers et al., 1984) and sequencing (Rogers et al., 1986) of a tRNA gene cluster (Rogers et al., 1987). A genome section containing the *rpoB* gene (Laigret et al., 1996), which codes for the β-subunit of RNA polymerase, also contains an *hsd* gene, which is possibly involved in type I restriction and modification systems, and phage sequences. The origin of replication, *oriC*, and the adjacent *dnaA* gene, responsible for the initiation of DNA replication, also were identified and sequenced (Ye et al., 1994). Gene organization in *S. citri* resembles that in a *Lactobacillus*, at least in the vicinity of the phosphofructokinase gene (Branny et al., 1993). The region also encodes the *rpsB* product (a ribosomal protein), elongation factor Ts (a component of the protein biosynthesis elongation cycle), spiralin, pyruvate kinase (Chevalier et al., 1990), and three other proteins (Le Dantec et al., 1998). Near the 3' end of the *rpsB* gene is a 20-bp inverted repeat that serves as a binding site for ribosomal protein L29, suggesting that the latter serves a regulatory role as well as a role in ribosomal structure and activity (Le Dantec et al., 1998). Numerous sequences have been found to be polymorphic among *S. citri* strains (Bové et al., 1990), and mechanisms to account for such polymorphisms have been proposed (Melcher et al., 1999b).

The first published report of investigation of *S. kunkelii* genome sequences was that of a survey sequence pilot project (Bai and Hogenhout, 2002a). Genome sequencing projects were initiated for *S. kunkelii* CR-2 in the United States (Zhao et al., 2003; Davis et al., 2004), and for *S. citri* GII3 in France (Gasparich, 2002; Foissac,

2004). Unannotated *S. kunkelii* sequences have been publicly available (http://www.genome.ou.edu/spiro.html {Spiroplasma kunkelii Strain CR2-3x Genome Sequencing Web site}) and the project is nearing completion with the assembly of the 1.55-Mbp genome. Annotations and analyses of partially assembled sequences have been published and include the characterization of an 85-kbp segment containing ribosomal protein genes (Zhao et al., 2003), ATP-binding cassette (ABC) systems (Zhao et al., 2004), and a cell division gene cluster (Zhao et al., 2004).

Only a few *S. citri* GII3 sequences have yet been made public, yielding information on glucose and trehalose permease operons (Andre et al., 2003). A search strategy identified four genes present in two phytopathogenic phytoplasmas and *S. kunkelii* but absent from selected *Mycoplasma* genomes (Bai et al., 2004b). These genes, encoding a polynucleotide phosphorylase, a CMP binding protein, and two less clearly identifiable proteins, may thus be involved in plant infection or insect transmission to plants (see the section Virulence Mechanisms in this Chapter).

Genetic Mechanisms

ULTRAVIOLET LIGHT. Spiroplasmas, particularly those in young cultures (Labarere and Barroso, 1989), are sensitive to UV radiation (Labarere and Barroso, 1984), and arsenic acid- and xylitol-resistant mutants of *S. citri* were recovered after UV treatment. Mutants are able to recombine when incubated for 90 min, suggesting that genetic exchange may be taking place.

GENE EXCHANGE. Spiroplasmas may exchange genes by a DNAse resistant mechanism (Barroso and Labarere, 1988). A recent electron microscopy study of *S. kunkelii* (Ammar et al., 2004) showed structures thought to be pili that could function in conjugation and DNA exchange (see section Morphology in this Chapter). Homologous recombination is thought infrequent in phytopathogenic spiroplasmas because of the absence of a complete copy of *recA* (Marais et al., 1996), a gene that, in most bacteria, functions in homologous DNA recombination and DNA damage repair. Nevertheless, recombination has been observed to occur in *S. citri* GII3 between an introduced replicating plasmid DNA and homologous sequences in the chromosome (Renaudin et al., 1995). Integration can occur at a targeted gene but will also occur in the *oriC* region owing to the presence of *oriC* sequences in the plasmid. Consistent with active recombination systems, integrated SpV1 phage sequences have been implicated in a variety of genome shuffling events (Melcher et al., 1999b). For example, *S. citri* BR3-3X has sustained a large inversion with accompanying small deletions (Ye et al., 1995b) and multiple insertions (Ye et al., 1996) during laboratory propagation.

INSERTIONAL MUTAGENESIS. Often employed to identify genes, insertional mutagenesis, may be done using intact transposons such as Tn*4001* or transposomes. After Tn*4001* mutagenesis of *S. citri* GII3, colony morphology screening resulted in the identification of a motility deficient spiroplasma (Foissac et al., 1997). The inactivated gene, designated "*scm1*," could be complemented with the wildtype gene (Jacob et al., 1997). Tn*4001* insertion in the *S. citri fruR* gene, which is located in the fructose operon, abolished the ability of *S. citri* to utilize fructose and also to produce disease (Gaurivaud et al., 2000; see the section Virulence Mechanisms in this Chapter). Tn*4001* insertion in an *S. citri* gene encoding a lipoprotein that is a solute-binding ABC transporter reduced transmission to plants by *C. hematoceps* (Boutareaud et al., 2004; see the section Insect Transmission in this Chapter). Transposomes, pieces of DNA with transposon ends to which transposase molecules are covalently attached, have been used to generate random, stable insertions in the genome of *S. citri* BR3-3X (Mutaqin et al., 2003; Mutaqin et al., 2004).

Extrachromosomal DNAs

Extrachromosomal DNAs in spiroplasmas (Renaudin, 2002) include plasmids and the replicative forms of bacteriophage genomes. Though found in many groups of spiroplasmas, including the phytopathogenic species in group I, extrachromosomal elements are not universal in spiroplasmas (Gasparich et al., 1993). Some species, for example *S. floricola*, have no detectable extrachromosomal elements.

PLASMIDS. Extrachromosomal closed circular DNAs exist in a variety of spiroplasmas in high abundance, up to 12% of the DNA mass (Ranhand et al., 1980). Characterized by electron microscopy, these DNA molecules were thought not to represent viral DNA forms. Plasmids (Harasawa and Barile, 1983) from a number of *S. citri* strains have been characterized (Bové et al., 1984), and their conservation and distribution among species are quite variable. For example, the distribution of one plasmid from *S. citri* strain ASP-1 was not universal among *S. citri* strains and spiroplasma species (Barber et al., 1983), and sequences similar to those of plasmid pM41 of *S. citri* (Mouches et al., 1984) have been found in many other, but not all, *S. citri* strains as well as in other spiroplasma species (Mouches and Bové, 1983a). Like those from other bacteria, spiroplasma plasmids may carry genes for antibiotic resistance. Transfer of

the erythromycin resistance of *S. citri* strain M4 Er-1 to *S. citri* R8A2 (by alkali cation plus polyethylene glycol treatment) was associated with transfer of a plasmid, pM42 (Salvado et al., 1989). Plasmids pBJS of *S. citri* BR3-3X (Joshi et al., 2004) and pSKU46 of *S. kunkelii* CR2-3X (Davis et al., 2004), each 13–14 kbp in size, contain genes for plasmid transfer, including *traE*, *mob* and *soj*. A pair of origins of transfer, *oriT*, have been identified in pSKU46, resulting in assignment of the plasmid to the IncP incompatibility group (Davis et al., 2004). Two of four *S. kunkelii* CR2-3X *traE* genes also are located on extrachromosomal DNA (Bai et al., 2004a). The *traE* gene of pBJS appears to be located at the end of a three-cistron operon, the other cistrons of which encode the adherence-associated membrane protein SARP1 and an unidentified seven transmembrane helix protein (Joshi et al., 2004). The pBJS plasmid is not present in all *S. citri* strains, but some strains lacking it have related plasmids, identified by DNA hybridization, that lack the *arp1* coding sequences. About three quarters of spiroplasma extrachromosomal elements are thought to contain SpV1 or SpV3 sequences (Gasparich et al., 1993) and may thus contribute to instability of these plasmids as cloning vectors. Interestingly, preliminary reports indicate that *S. citri* strain GII3 has six plasmid DNAs, of which five contain one or more *arp1* homologues, for a total of eight such genes (Foissac et al., 2004). Apparently, strains of phytopathogenic spiroplasmas differ widely in their contents of these plasmids.

VIRUSES. Soon after their discovery, phytopathogenic spiroplasmas were shown to be infected with phages (Cole et al., 1973). The diversity of mollicute-infecting phages (Maniloff et al., 1977; Jansson et al., 1982; Maniloff, 1988) suggests that spiroplasmas are unusual among the class in being frequent phage hosts. Phytopathogenic spiroplasmas host at least four kinds of phages, designated SpV1 (Renaudin and Bové, 1994), SpV2 (Cole et al., 1973), SpV3 (Cole et al., 1977), and SpV4 (Renaudin and Bové, 1994). Not all non-phytopathogenic spiroplasma species harbor phages, however (Chastel et al., 1987). Indeed virus-related sequences, as determined by Southern blotting, are limited to group I spiroplasmas.

SpV1-like phages have been observed in many spiroplasmas (Liss and Cole, 1982). Each spiroplasma strain is resistant to the SpV1 phage it harbors but is susceptible to others (Liss and Cole, 1982). These phages are plectroviruses in the family Inoviridae and have properties associated with the family: they are rod-shaped, packaged by budding from the cell surface, and have single-stranded DNA in the particles with an intracellular double-stranded DNA replica-

tive form (RF). The first SpV1 phage genome to be completely sequenced, SpV1-R8A2 B (Renaudin et al., 1990), encodes an IS30 family insertion sequence (Dong et al., 1992). SpV1-C74 (Renaudin, 1996), in contrast, encodes a transposase characteristic of the IS3 family (Melcher et al., 1999b). Both circular phage genomes have inverted repeat sequences characteristic of their insertion sequence family. These repeats are adjacent but separated by a spacer whose length is also a characteristic of the family to which the elements belong. Thus, the phage genomes resemble circular DNA IS element transposition intermediates. The SpV1-like phage SVTS2, isolated from the group I spiroplasma *S. melliferum,* also infects *S. citri.* Traces of its genome in *S. kunkelii* suggest that it infects this species also. Its complement of genes resembles those of the *S. citri* phages, except for the transposases, which are replaced in SVTS2 by a replication protein cistron (Sha et al., 2000).

SpV2 viruses, tailed phages in the Siphoviridae, have been observed by electron microscopy but neither propagated nor further characterized (Cole et al., 1973). An *S. citri* membrane protein, P58 (Ye et al., 1997), identified because of its deletion during a rearrangement that led to a leafhopper nontransmissible strain (Ye at al., 1996), has convincing similarity in amino acid sequence to terminases encoded by phages of the Siphoviridae. Southern hybridization revealed multiple copies in almost all strains of *S. citri* and *S. kunkelii* tested. For example, at least half a dozen full or partial diverged copies of the P58 gene occur in the *S. kunkelii* CR2-3X genome (J. Comer et al., unpublished observations). The copies appear to have resulted from recombination between two parental sequences. Homologues of the P58 gene also are present in the *Ureaplasma parvum* genome, where they and several flanking genes that are also homologous to genes in *S. citri* and *S. kunkelii* occur in the same order as they do in the latter two species (J. Comer et al., unpublished observations). On the other hand, no P58 copies were detected in other *Spiroplasma* species, mycoplasmas, or phytoplasmas. The observations are consistent with these genes being remnants of infection of plant phytopathogenic spiroplasma by at least two SpV2-like phages distantly related to a ureaplasma phage. Alternatively, the genes could be the result of an ancient infection of an early mollicute ancestor, followed by loss of the genes from all but a few of the derived lineages.

SpV3 phages are poyhedra with short tails (Cole et al., 1977). They have been found in *S. citri*, the suckling mouse cataract agent *S. mirum* (Cole et al., 1977), and the *Drosophila* sex ratio organism, *S. poulsonii* (Cohen et al., 1987). An incomplete form of the genome of ai, an SpV3

phage, was found in the genomes of all strains of *S. citri* examined (Dickinson and Townsend, 1984). The absence of sequence information for these phages precludes identification of their prophages in the *S. kunkelii* CR2-3X sequence.

Virions of SpV4, a member of the Microviridae (Renaudin et al., 1984), resemble phiX174 particles except that they have 20 protrusions extending from the icosahedron, whereas phiX174 virions have no protrusions (Chipman et al., 1998). The genome sequence of SpV4 was the first complete mollicute virus sequence determined (Renaudin et al., 1987a; Renaudin et al., 1987b) and analyzed (Renaudin et al., 1987c). The identification of transcription start and stop sites on SpV4 DNA led to the identification of probable promoter and terminator sequences active in phytopathogenic spiroplasmas (Stamburski et al., 1990). That *S. citri* could be transformed with exogenous DNA was first demonstrated with SpV4 (Bové et al., 1984).

Viral nucleic acid sequences are frequently found integrated in the spiroplasma chromosome (Renaudin et al., 1990). Probably through repeated cycles of phage infection, integration and divergence, the genomes of *S. kunkelii* and *S. citri* have accumulated many degenerated copies of SpV1-like phages. Representatives of each SpV1-R8A2 B, SpV1-C74 and SVTS2 phages are present. Terminal sequences of phage genomes integrated in the *S. kunkelii* genome actually suggest that two distinct phages related to each SpV1-R8A2 B and SpV1-C74 have participated (U. Melcher and J. Fletcher, unpublished observation). A remarkably high percentage (10–20%), of the genomes of phytopathogenic spiroplasmas consists of phage-derived sequences. Early reports that plasmid sequences are found, by Southern blotting, in chromosomal DNA of *S. citri* and other spiroplasma species (Mouches et al., 1984) may indicate that these extrachromosomal DNAs were RFs of phages. Independent recombination events eliminating the degenerated viral sequences prevent the genome from indeterminately expanding (Melcher and Fletcher, 1999a). However, genome sizes can expand rapidly during laboratory propagation (Ye et al., 1996), presumably because of continuing integration events.

Viral integrations may be neutral or beneficial to the spiroplasmas. For example, integration events may lead to gene inactivation (Melcher et al., 1999b), and subsequent recombination events can result in inversions of large parts of the chromosome, both during speciation and during laboratory propagation (Ye et al., 1996). Other integration events provide host gene regulatory signals. For example, transcription termination in the fructose operon of *S. citri* has been inferred to occur at a terminator supplied by a phage sequence at the end of the *fruK* gene (Gaurivaud et al., 2000). In the case of spiroplasma viruses, integration may confer immunity to subsequent infection by that virus or a closely related one. Resistance of natural mutants of *S. citri* to infection with SVTS2 was inferred to reflect classical phage immunity (Sha et al., 1995) since electroporation of the viral RF into cells did not initiate an infection, and the mutant strains contained integrated viral sequences not present in the parent. On the other hand, that *S. citri* strain R8A2 is susceptible to phages SpV1-R8A2B and SpV1-C74 (Renaudin, 2002), even though its genome contains phage-related sequences, can be attributed to evolutionary divergence of viral sequences after integration, leading to inactivation of the immunity factor.

A)

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TGTGTGGTATACTACA.TTTGTAGAAACCACATACATTTT
CCGTATGATTTACTAAAAATGTAGAAACCACACACATTTT
TTTGTGGATTATTTTC.CTTCTATAAACCACACACATTTT
ATCTTCTAATTTT.....TTGTAGAAACCACACACATTTT
TAGAAGAAATGATT.A.ATTGTAGAAACCACACACATTTT
GTTAAATCAAAAGAAATAGTGTAGAAACCACACACATTTT
ATGGTGTGGGGTATCAATTTGTAGAAATTACACACATTTT
ATGAATTTAATTATAAATGTGTAGAAACCACACACATTTT
CCGCCTTGATGAC......TGTAGAAACCACACATTTT
CATTAGTTATAACTACTAGTATAGAAACCACACACATTTT
ATCAATTTAATTATAAATGTGTAGAAACCACACACATTTT
GTTGAGTGAAAATATTAACTGTAGAAACCACACACATTTT
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B)

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ATTAAAATTATTTTAA..TTGTAGATTGTAAATGCCAACC
CTTTTCATTATAATAAATTTGTAGATTGTAAATGCCAACC
..........TTAAGT..TTGTAAATTGTAAATGCCAACC
CTTCTAATTCTTTCTAG..TGTAAATTGTAAATGCCAACC
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Fig. 6. Left ends of two groups (A and B) of SpV1-C74-like sequences in the *S. kunkelii* genome. Red font shows the conserved end sequences. Black font distinguishes point mutations in the ends and the nonrelated flanking sequences. Two distinct groups were also found for SpV1-R8A2 B-like sequences.

Molecular Genetic Methods

TRANSFORMATION AND TRANSFECTION. That *S. citri* could be transformed with exogenous DNA was first demonstrated with the DNA spiroplasma phage SpV4 (Bové et al., 1984), but the use of plasmids as vectors has generally been more successful than the use of viruses. For example, when erythromycin resistance was transferred from *S. citri* strain M4 Er-1 to *S. citri* R8A2+ by alkali cation plus polyethylene glycol treatment, the transfer was associated with transfer of plasmid pM42 (Salvado et al., 1989). Polyethylene glycol also induced fusion between pairs of mollicutes, including spiroplasmas (Tarshis et al., 1991). Fusion of

spiroplasmas with large unilamellar vesicles containing DNA has been used for transformation of *S. floricola*, a non-phytopathogenic mollicute that colonizes flower surfaces (Salman et al., 1992; Salman et al., 1993). Transformation by conjugal transfer from species such as *Enterococcus faecalis* has been reported for *Mycoplasma* species but not for any *Spiroplasma* species (Renaudin et al., 2002).

Electroporation now serves as a standard way of transfecting spiroplasma with phage DNA (Gasparich et al., 1993). Electroporation (McCammon et al., 1990) of a plasmid bearing transposon Tn*4001* resulted in insertion of the transposon in *S. citri* strain GII3 (Foissac et al., 1996) but not in several other strains (Foissac et al., 1997). A wide variation in the susceptibility of *S. citri* strains to electroporation-mediated transformation may be due to DNA methylation (McCammon et al., 1990). Transfection has been used in other experimental applications: for example, to demonstrate that the RF of SpV4, cloned in an *E. coli* plasmid vector, was infectious after restriction and circularization (Pascarel-Devilder et al., 1986). *Spiroplasma citri* transformed with a plasmid bearing a *Mycoplasma* sequence was used as an internal control for detecting the mycoplasma by PCR (Verdin et al., 2000).

MARKERS AND SELECTION. The successful construction of transformation vectors requires the availability of marker genes for selection. *Spiroplasma citri* mutants resistant to arsenic acid, vanadium oxide, and xylitol have been isolated (Barroso and Labarere, 1988; Barroso et al., 1990), but of these only xylitol resistance was useful in selection schemes. The chloramphenicol acetyltransferase gene, when introduced using SpV1-R8A2 B RF as a vector, was expressed in spiroplasma (Stamburski et al., 1991), but other antibiotic markers have proven more useful (S. Duret and J. Renaudin, cited in Renaudin, 2002). Resistance to tetracycline and gentamycin, encoded by the *tetM* gene of Tn*916* and the transposon Tn*4001*, respectively, have been used to select transformants of *S. citri* (Stamburski et al., 1991; Foissac et al., 1997).

VECTOR CONSTRUCTION. Genetically useful transformation of spiroplasmas also required the development of vectors capable of stable, independent replication in spiroplasmas. Vector development has proven challenging. In an effort to construct a plasmid vector, pCT-1 (an 11-kb extrachromosomal element from the non-phytopathogenic *S. taiwanense*) was characterized by restriction mapping and cloned in pBR322 (Gasparich and Hackett, 1994). The replicative form of the SpV1 phage was used as a vector to try to express a foreign gene in *S. citri* (Marais et al., 1993), but expression was complicated by recombination of the vector with

chromosomally located sequences (Marais et al., 1996).

Inactivation of *S. citri* genes by homologous recombination has been accomplished via the development of specific vectors such as the plasmid pBOT1, which is capable of replicating in *E. coli* (Renaudin et al., 1995). Owing to the presence of a spiroplasma chromosomal origin of replication, *oriC*, pBOT1 also replicates in *Spiroplasma*. It was tested for the ability to inactivate a gene required for motility (Duret et al., 1999), previously inferred to exist by the creation of motility mutants following nitrous acid treatment of *S. melliferum* (Cohen et al., 1989). One problem with pBOT1 as a gene inactivation vehicle was the presence of a large segment containing *oriC*. This region was itself susceptible to homologous recombination, leading to integration of the plasmid not in the target gene but in the *oriC* region. In an improved vector, pC2, the *oriC* region was trimmed to contain only the sequences needed for its replication (Lartigue et al., 2002). The resulting plasmid is active in several mycoplasma species as well as in spiroplasmas (Lartigue et al., 2003). One derivative, pC1/2, retains the ability to replicate in *S. citri* but appears to have lost the ability to recombine with the homologous chromosomal sequence (S. Duret and J Renaudin, cited in Renaudin, 2002).

pBOT1 has proven useful in producing gene addition lines. The additions usually are used to test whether an isolated gene provides the function missing in a mutant line. For example, insertion via an *oriC* vector of the fructose operon restored ability to use fructose and pathogenicity to a Tn*4001* nonpathogenic mutant (Gaurivaud et al., 2000). Similarly, insertion of a wildtype *scm1* gene restored motility to a mutant in which that gene had been disrupted (Jacob et al., 1997).

EXPRESSION OF SPIROPLASMA GENES IN OTHER ORGANISMS. Despite the codon differences between spiroplasmas and other bacteria (see the section General Characteristics in this Chapter), spiroplasma genes have been expressed in other organisms. An attempt was made to express the *S. citri* spiralin gene from a *Lactobacillus* plasmid in *Acholeplasma laidlawii* (Jarhede et al., 1995), but the spiralin synthesized was not processed and was made in lower than expected amounts. Plasmid pMH1 from *S. citri* has been cloned in *E. coli* as a chimera with pBR328 (Mouches et al., 1983b).

Insect Transmission

Leafhopper Feeding Behavior

Spiroplasmas and other phloem-restricted mollicutes are introduced into phloem when

inoculative vectors pierce the sieve element and subsequently salivate into the cell. This process is quite complex; successful phloem ingestion is the culmination of a series of events.

To acquire and then inoculate plant pathogenic spiroplasmas, leafhoppers must negotiate their flexible stylets through plant tissues and ultimately locate the phloem. Once a leafhopper lands on a potential host plant, the labrum is brought into contact with the plant surface. Mechanical and gustatory cues coming from mechano- and chemosensory organs assist the insect to orient over a vein. Small quantities of sheath saliva are secreted onto the plant surface through which plant chemicals diffuse and are detected by the insect's chemosensilla (Backus, 1985). The stylet tips are inserted into the plant epidermis and small quantities of plant cell contents are imbibed. If the cues are acceptable, the mandibular stylets remain anchored in the epidermis while the inner maxillary stylets continue to move deeper into the plant.

Leafhoppers are highly plastic with respect to the ability to probe and ingest from different plant tissues. With their stylets following an intracellular pathway on their way to the vascular tissues (Backus, 1985; Wayadande and Nault, 1993; Lett et al., 2001), deltocephaline leafhoppers have been documented, using electronic monitoring technology, to probe epidermis, mesophyll, phloem and xylem (Wayadande and Nault, 1996; Lett et al., 2001). Although relatively few species have been so monitored, some important spiroplasma vectors, *Dalbulus* spp., *Graminella* spp. (Wayadande and Nault, 1993) and *C. mbila* have been shown to display highly plastic host tissue preferences. These leafhoppers feed primarily from phloem tissues, but stylet pathways to the phloem for individual leafhoppers were often circuitous. Individuals ingested sap from other tissues for long periods before initiation of phloem contact (Wayadande and Nault, 1996). This observation suggests the possibility that phloem location behavior could be altered through plant breeding or genetic manipulation, as was accomplished for the rice host of the phloem-restricted plant virus, rice tungro (Khan and Saxena, 1985; Rapusas and Heinrichs, 1990; Wayadande, 1992).

Inoculation behavior has not been documented for spiroplasmas to the extent that it has been for other phloem-restricted pathogens, including barley yellow dwarf luteovirus (Scheller and Shukle, 1986), maize chlorotic dwarf waikavirus (Wayadande and Nault, 1993), and tomato yellow leafcurl geminivirus (Jiang et al., 1999). Presumably, because spiroplasmas are specifically found in phloem sieve elements, they are deposited there by their leafhopper vectors by salivation during phloem contact. Deposition in any other plant tissue and subsequent phloem

invasion would require that spiroplasmas have the ability to penetrate the phloem cell wall, an event that has been neither documented nor suggested. The study of phytopathogen inoculation by leafhoppers has been greatly enhanced by electronic monitoring technology. Now more widely known as electrical penetration graph (EPG) technology, a more intimate understanding of feeding activity *in situ* at the stylet tips is now possible. EPG monitors detect minute fluctuations in electrical current passing through food or salivary canals of feeding hemipterans, and represent them as waveform patterns. Two EPG patterns have been correlated with phloem probing: the x-waveform (a term that is no longer used) and the phloem ingestion pattern. The x-waveform, which has been documented for several deltocephaline leafhoppers, including several spiroplasma vectors, was suggested to represent alternating bouts of salivation and ingestion (McLean, 1977; Wayadande and Nault, 1993). Spiroplasma inoculation activity has not been specifically studied, but leafhopper salivation into the phloem sieve element has been recently documented for the *S. kunkelii* experimental vector, *C. mbila*, by EPG (Lett et al., 2001). Using a DC monitoring system, Lett et al. (2001) showed, using AC monitoring, that patterns 4 and 5, which correspond to the x-waveform and phloem ingestion patterns, are specifically associated with feeding activity in phloem sieve tubes. Furthermore, they showed that pattern 4 (x-waveform) contains components representing salivation activity. Thus, salivation into the phloem sieve tube during pattern 4 activity is the time at which spiroplasma inoculation is most likely to occur.

Spiroplasma-Leafhopper Interactions

SPIROPLASMA MOVEMENT THROUGH VECTOR. Leafhopper-transmitted spiroplasmas propagate in their vectors, requiring a 17–23-day latent period between acquisition and inoculation (Nault, 1980). Spiroplasmas are ingested during acquisition feeding, moving into the lumen of the alimentary canal. Kwon et al. (1999) showed that *S. citri* moved into the gut epithelium of the anterior midgut (filter chamber) of the vector *C. tenellus* (Fig. 5). Spiroplasmas were observed attached to the base of microvilli and in small membrane-bound vesicles near the apical plasmalemma. This same observation was made by Ammar et al. (2004) and Ozbek et al. (2003) in *Dalbulus* spp. vectors of *S. kunkelii.* Interestingly, the number of spiroplasmas observed in *S. kunkelii*-infected *Dalbulus* were much greater than observed in *S. citri*-infected *C. tenellus*, possibly reflecting the lower transmission rate observed for *S. citri* (Way-

adande and Fletcher, 1995) compared to that of *S. kunkelii* (Markham, 1983a).

Movement through insect tissues was suggested to be mediated by spiroplasma adhesins (Fletcher et al., 1998; Kwon, 1999). Spiroplasmas attach to the apical plasmalemma, initiating invagination into gut or salivary gland cells (Liu et al., 1983; Kwon et al. 1999). Recent micrographs of *S. kunkelii* invading *Dalbulus* gut epithelial cells show the presence of tip structures (Ammar et al., 2004) similar to those of *Mycoplasma* spp. that mediate host cell invasion (Rottem, 2003). A core of electron dense material at the center of the tip structure is clearly visible when spiroplasmas are in contact with insect plasmalemma, but less so once spiroplasmas are completely engulfed. Ammar et al. (2004) and Ozbek et al. (2003) also report the presence, on the surface of *S. kunkelii*, of pili-like structures, which in other bacteria serve as surface attachment structures (see the section Morphology in this Chapter). How spiroplasmas cross the basal lamina of gut epithelial cells to reach the hemolymph and again at the salivary gland-hemolymph interface has been the subject of much speculation. Kwon et al. (1999) showed that *C. tenellus* basal lamina becomes degenerated and less coherent as a result of spiroplasma infection, suggesting that the pleomorphic spiroplasmas could squeeze through to the hemolymph. Ozbek et al. (2003), however, presented micrographs clearly showing breaks in *D. maidis* basal lamina, and spiroplasmas apparently moving through to the hemolymph.

Spiroplasma adhesion and movement into vector host cells is thought to be mediated by cell surface proteins. *Spiroplasma citri* line BR3 protein P89, later designated "SARP1," was implicated in cell surface adhesion to vector cell line cells, an event that was blocked by prior treatment of spiroplasmas with the proteases trypsin and proteinase-K and restored after removal of the enzymes (Yu et al. 1998). The amino acid sequence of SARP1 was similar to that of a known adhesin of *Mycoplasma agalactiae* (Fleury et al., 2002). Interestingly, versions of SARP1 are found on both the chromosome and a 13-kb plasmid of *S. citri* (Joshi et al., 2004), and also in *S. kunkelii* (Davis et al., 2004). The N-terminal regions of SARP1 consist of a series of six or seven 40-residue repeats, designated "sarpins," that are found in a scattered diversity of other bacterial proteins (Berg et al., 2001). Although line BR3 has only a single plasmid bearing the *arp1* gene (Joshi et al., 2004), Foissac et al. (2004) recently showed that line GII3 has six plasmids, five of which contain a total of seven *arp* homologs. Berho et al. (2004) recently showed that the GII3 SARP protein P82 (SARP4a) had strong similarity to the BR3

SARP1. Direct evidence that the SARP proteins are directly involved in transmission await gene inactivation and complementation studies.

Other spiroplasma proteins are also implicated in the transmission process. As suggested by Boutareaud et al. (2004), the movement of spiroplasmas through the insect (crossing gut barriers, multiplication in the vector tissues, and crossing salivary gland barriers) likely involves several spiroplasma proteins. The requirement for adhesin-type proteins is well documented for several *Mycoplasma* spp. (Rottem, 2003). Other transmission related proteins recently identified include the GII3 plasmid-encoded protein, P32 (which is present on both transmissible and poorly transmissible *S. citri* lines but absent from nontransmissible lines; Killiny, 2004) and a solute binding protein of an ABC reporter (Boutareaud et al. 2004). Finally, spiralin, the dominant spiroplasma surface protein, was recently shown to be required for transmission (Duret et al., 2003). Although the mechanism of spiralin involvement is unknown, its ubiquitous distribution on the outer membrane of spiroplasmas (Morley, 1988) is consistent with a possible adhesion function. This putative function was recently supported by Killiny et al. (2004) who demonstrated that isolated spiralin protein binds to glycoproteins, a feature common among adhesins. However, other proteins are very likely involved, as loss of transmission has not always been correlated with presence or absence of spiralin. Natural *S. citri* mutants BR3-G and BR3-P, which are no longer insect transmissible or are very poorly transmitted, still express spiralin (Fletcher et al., 1995). The functions of intact spiralin and these other proteins and their specific roles in the transmission process are important foci of ongoing studies.

There have been no reports of vertical transmission of spiroplasmas to the offspring of infected females, but there have been a few reports of transovarially transmitted phytoplasmas. Alma et al. (1997) and Hanboonsong et al. (2002) both report vertical transmission of aster yellows and sugarcane white leaf phytoplasmas, respectively, through infection of leafhopper eggs. Detection of these phytoplasmas in eggs and resulting offspring was by nested PCR. This type of detection always carries the possibility that the egg was contaminated with phytoplasmas adhering to the chorion, but the possibility of transovarial transmission of mollicutes remains intriguing. Convincing demonstration of spiroplasma transovarial transmission will likely include PCR detection, transmission of spiroplasmas by offspring isolated as eggs, and transmission electron micrographs of spiroplasmas within the ova.

SPIROPLASMA PATHOGENICITY TO INSECT HOSTS. Spiroplasma infection may have an impact upon the physiology of the vector. After acquisition by the leafhopper, spiroplasma (both *S. citri* and *S. kunkelii*) titers dropped initially, but rebounded in vivo, reflecting multiplication within insect hosts. Spiroplasmas or spiroplasma-like bodies were seen in various tissues including gut epithelia, hemolymph, Malpighian tubules, fat body, neural tissue, muscle, and salivary glands (Liu et al., 1983; Markham, 1983a; Kwon et al., 1999; Osbek et al. 2003; Ammar et al. 2004). Negative impacts of *S. citri* on its vector *C. tenellus* included severe cytopathology of insect tissues (Kwon et al., 1999), including degeneration of salivary gland cells, disorganization of muscle fibers, and compromised cell integrity. In addition, leafhopper longevity and fecundity were reduced after acquisition of *S. kunkelii* (Granados, 1975; Vega et al., 1975; Ebbert and Nault, 1994). These observations have not been coupled with reports of significantly reduced vector competency or flight activity, however. In fact, early reports of the impact of spiroplasmas on their vectors suggested that pathogenicity was slight or nonexistent after injection (Whitcomb et al., 1974). There may even be some benefit to an insect carrying a population of spiroplasmas. Ebbert and Nault (2001) demonstrated that *S. kunkelii*-infected *D. maidis* were able to withstand drier overwintering conditions than were their uninfected counterparts. So, although spiroplasma infection may be detrimental to individuals under optimal growth conditions, environmental stressors may shift the selective advantage to infected populations.

Epidemiology

Factors affecting the spread and distribution of insect-transmitted plant pathogens include vector abundance, proportion of the population capable of transmitting the pathogen, and plant-to-plant dispersal (Irwin and Ruesink, 1986). The primary vectors of both *S. citri* and *S. kunkelii* (Table 1) are abundant in regions where these pathogens are prevalent, becoming more so as reproductive plant hosts become more available year round. Spiroplasma-susceptible plant hosts are more likely to serve as feeding hosts in areas where inoculating vectors occur in higher numbers as opposed to areas with low vector densities. What motivates inoculative vectors to move to a new host? The propensity of the spiroplasma vectors to move from one plant to another is affected by several factors including plant homogeneity (Power, 1988; Power, 1992; Castro et al., 1992), plant spacing (Power, 1992), diel periodicity (Kersting and Baspinar, 1995), and vector

gender. Studies of spacing and composition of spiroplasma hosts within a field indicate that closely spaced, homogeneous plantings are more likely to promote insect movement. Power (1988) suggested that genetically diverse plantings of maize were less attractive than pure stands to colonizing *Dalbulus* leafhoppers. This phenomenon may have resulted from the genetically diverse stands being suboptimal reproductive hosts or from greater emigration. Within monocultures, leafhoppers move more frequently between closely spaced plants (Power, 1992). This is consistent with the findings of Castro et al. (1992), who showed greater flight activity within maize monocultures than in maize-bean bicultures. They also compared *S. kunkelii* incidence with maize densities but found few differences.

Diel periodicity (flight activity during a 24-h period) and gender may also influence spiroplasma spread throughout a field. For example, males of the *S. kunkelii* experimental vector *Graminella nigrifrons* transmit the maize chlorotic dwarf waikavirus more readily than do females, primarily because males move from plant to plant in search of virgin females. In this "call-fly strategy," males communicate acoustically with females on adjacent plants. Males fly short distances, intermittently calling and feeding on virus-susceptible tissues (Hunt et al. 1993). This strategy was suggested to explain the fact that sticky traps placed in sesame plantings sustained higher numbers of male *C. haematoceps* (the European vector of *S. citri*) than of females, indicating greater male crepuscular flight activity (Kersting and Baspinar, 1995). Since many leafhoppers, including those in the genus *Dalbulus*, exhibit similar acoustic behavior (Heady and Nault, 1986), the call-fly strategy likely influences *S. kunkelii* spread by male, inoculative *D. maidis*.

The *Circulifer* vectors of *S. citri* are polyphagous, feeding on a number of annual and perennial hosts, many of which are *S. citri* plant hosts (Oldfield, 1988). Lowered *C. tenellus* discrimination among plant hosts or simply the insects' ability to locate and feed from the phloem of a number of plant species, such as citrus, that are poor long-term maintenance hosts (Cook, 1967), may explain, in part, why this species is able to inoculate *S. citri* to such a wide range of plant hosts. The genus *Circulifer* contains the best-documented vectors of *S. citri* and is one of the most important vector taxa because other pathogens transmitted by this group, most notably beet curly top geminivirus, are very significant (Klein, 1992). However, other *S. citri* vector species also deserve continued attention. Although most initial infections of citrus may occur by *C. tenellus* feeding after acquisition from weed hosts,

within-grove (citrus to citrus) spread may involve leafhoppers of the genus *Scaphytopius*, which reproduce on citrus (Oldfield, 1988). Early studies of the contributions of *Scaphytopius* spp. in *S. citri* epidemiology have not been continued because of limited resources and personnel working with these spiroplasma vectors.

Disease

Symptoms

Spiroplasmas incite at least three economically important plant diseases: corn stunt, caused by *S. kunkelii,* and citrus "stubborn" and brittle root disease of horseradish, caused by *S. citri*. *Spiroplasma phoeniceum*, a third plant pathogenic species, was isolated from periwinkle plants placed in a citrus orchard to serve as sentries for *S. citri*. The periwinkle plants developed symptoms similar to those induced by *S. citri*, but spiroplasmas cultured from such plants proved to be a new species (Saillard et al., 1986). Although *S. phoeniceum* has since been characterized, its natural host(s) remain cryptic.

Plant phloem presents a highly specialized environment for growth and multiplication of spiroplasmas. These mollicutes colonize and multiply to high titers in plant host sieve tubes, comprised of vertical columns of sieve cells, specialized living cells that lose their nuclei upon maturation and rely on adjacent phloem companion cells for various nucleus-directed activities. Porous sieve plates separate one sieve cell from another within each column, and spiroplasmas pass readily through these pores. They are probably carried along with photosynthates, which are produced in the chlorophyll-rich leaves and stems and translocated through the sieve tubes to non-green "sinks," plant parts such as flowers, fruits, storage organs, roots and expanding leaves (Kloepper et al., 1982). For example, *S. citri*, inoculated into turnip seedlings by confining inoculative *C. tenellus* on designated leaves, were recovered from roots four days before they were found in noninoculated leaves (Fletcher and Eastman, 1984a). Similarly, when inoculative *D. maidis* were confined on the first true leaf of corn seedlings, *S. kunkelii* moved into roots, developing leaves, and tassels over time (Gussie et al., 1995). Spiroplasma titers are often highest in actively growing tissues such as shoot tips. The phloem environment is highly suitable as a spiroplasma growth medium, providing both nourishment and an osmotically suitable milieu for these wall-less (and thus osmotically fragile) microbes.

CORN STUNT. This is one of the most costly diseases of *Zea mays* in countries of the Western Hemisphere (Nault et al., 1979). Although the condition originally was attributed to a single virus, it is now known to be a disease complex in which *S. kunkelii,* one or more of several viruses (maize rayado fino and maize chlorotic dwarf viruses), and a phytoplasma (most of which are transmitted by the same leafhopper vectors) may be involved. What was known as the Rio Grande form of the disease (Maramorosch, 1955) is now recognized as the syndrome produced by *S. kunkelii* infection. The so-called "Mesa Central" version of corn stunt, now known as "maize bushy stunt," is caused by a phytoplasma of the same name (Nault, 1980). Symptoms of the spiroplasma-induced stunt disease, which appear about 6 weeks after infection, include the appearance of small chlorotic stripes at the leaf base. The stripes lengthen and widen, eventually merging together. Symptoms vary in nature and intensity depending on the time of inoculation. In maize cultivars normally having significant levels of reddish anthocyanin pigments, irregular reddish or purplish areas may be present. Affected plants have shortened internodes, giving rise to the "stunt" phase of the disease. Proliferation of lateral shoots and roots, and excessive tillering, give the plants a bushy appearance. The numbers of ears may be greater, but they are smaller and often are sterile or have very few kernels. These symptoms may be altered or masked, however, in the case of mixed infections of *S. kunkelii* with the other pathogens mentioned above.

CITRUS STUBBORN. The name of this disease was coined in 1921 by a citrus grower frustrated with the slow growth of healthy buds grafted onto symptomatic trees (Calavan and Bové, 1989). Symptoms elicited in citrus by *S. citri* vary with the host variety and environmental conditions but generally are more pronounced on young trees (most trees become symptomatic within 5–6 years of planting) and under high temperature conditions (Calavan and Bové, 1989). The impact may be mild initially, but trees grow more slowly and become stunted compared to healthy trees of the same age planted nearby. Shortened internodes, multiple buds, and prolific shoot growth give the tree a dense, bushy appearance. New leaves are often smaller in size (the disease in Israel has been called "little leaf"), may be deformed (with a curled and mottled appearance), and may drop prematurely. Twig die-back and bark thickening are common. Like the leaves, flowers too may be reduced in size, although they are of normal appearance and color, lacking any form of the virescence (petal greening) or phyllody (leaf-shaped petals) that are commonly seen in phytoplasma-induced diseases. Fruits are often crooked and elongated, with a curved axis, and

Fig. 7. Horseradish (*Armoracea rusticana*) showing symptoms of brittle root disease caused by *Spiroplasma citri*. Panel A: foliar chlorosis and necrosis. Panel B: field of affected horseradish in southern Illinois during 1979 epidemic. Panel C: sections of diseased root showing phloem necrosis. Photos: C. E. Eastman (A, B) and N. Simkus (C), Illinois Natural History Survey, Champaign, IL.

the peel may be thinner than normal or thick and "cheesy." Greening of the style end is common, and "blue albedo," a bluish discoloration in the albedo of the peel, is sometimes seen. Seeds of affected fruits may abort. Flowers and fruit may be more numerous on affected trees, but early fruit abscission causes reduced yield. In advanced stages, affected branches wilt and the tree becomes weakened and highly vulnerable to other pathogens and stresses.

HORSERADISH BRITTLE ROOT. This disease is first apparent as foliar chlorosis, with leaves often bright golden yellow (Fig. 7). Young leaves may be stunted, leaf edges may curl and become necrotic, and leaf asymmetry may occur. The entire plant becomes stunted, wilting may occur, and foliage ultimately turns brown and decays. Root cross-sections show a yellowish-tan internal ring, caused by necrosis of the phloem, and take on a brittle quality from which the disease name is derived (Fletcher et al., 1981). Plants infected late in the season may remain symptom-free, but root pieces planted the following year usually develop brittle root symptoms and die soon after foliar emergence. A devastating epidemic in the late 1970s caused unprecedented losses and prompted an intensive

research initiative culminating in the completion of Koch's postulates demonstrating the causal agent to be *S. citri* (Fletcher et al., 1981; Raju et al., 1981b).

Losses and Impact

CORN STUNT DISEASE. The condition now recognized as corn stunt disease was first reported in the Rio Grande Valley of Texas by G. E. Alstatt in 1945 (Alstatt, 1945) and became widespread and very destructive throughout the Neotropics where corn is grown. The fact that multiple pathogens could co-infect maize (see the section Symptoms: Corn Stunt in this Chapter), causing variations in symptom syndromes and impact, caused some confusion about disease etiology, geographical range, and epidemiology during the 1950s–1970s. However, the spiroplasma-incited corn stunt disease is now known to occur in all countries where the vector occurs, from the southern United States to Argentina (Whitcomb, 1989), and is one of the most costly maize diseases in those locations (Bradfute et al., 1981; Tsai and Falk, 1988). In Texas, as much as 30% of corn fields were affected in the 1940s (Alstatt, 1945), and in Flor-

ida, a pathogen complex including *S. kunkelii* led to the loss of hybrid seed production and breeding nurseries, a $60 million enterprise, in 1979 and 1980 (Bradfute et al., 1981). Losses in the tropics can be even higher, nearing 100% in severe epidemics (Whitcomb, 1989).

CITRUS STUBBORN DISEASE. Unproductive navel orange trees showing symptoms of stubborn disease were first noted during a 3-year study (1915–1917) near Redlands, California, but the disease was so prevalent at that time that the pathogen probably was present in the United States by the turn of the century (Calavan and Bové, 1989). The first published disease description, however, came in 1928 when Reichert reported an epidemic of citrus "little-leaf" disease in Palestine. Again, the death of thousands of trees in this epidemic suggests that the pathogen had been present for a number of years. In the late 1950s and early 1960s, citrus stubborn had reached huge acreages of orchards, including many citrus species, in California. By then, Calavan (1969) estimated the number of diseased trees in California alone at about a million. The disease continued to spread, ultimately affecting from 50–100% of the trees in some orchards, and in total over 2 million trees were lost (Lee et al., 1973). The range of citrus stubborn also continued to increase in the Mediterranean region during this period, affecting many citrus species in Morocco, Algeria, Tunisia, Lebanon, Syria, Turkey and Corsica during the 1950s. By the late 1960s, it had been diagnosed in most countries of northern Africa and the Middle East (Calavan and Bové, 1989).

HORSERADISH BRITTLE ROOT. Horseradish, *Armoracia rusticana*, is a specialty crop produced commercially in southern Illinois (Fletcher et al., 1981; Fletcher, 1983). Reported first in 1936, brittle root disease has occurred sporadically in subsequent years and is considered the most important disease of the crop. Although its incidence is usually fairly low, it occasionally reaches epidemic proportions, causing 100% losses for individual growers and 25–80% loss to the horseradish industry in the state. In 1983, the disease was found in low incidence in horseradish fields in Maryland (Davis and Fletcher, 1983), expanding significantly the geographical range of the pathogen.

Virulence Mechanisms

The mechanisms by which spiroplasmas colonize and cause disease symptoms in host plants may be complex, involving a number of different virulence strategies.

PHLOEM DYSFUNCTION. Some of the symptoms induced by spiroplasma colonization of sieve tube elements reflect interruption of normal phloem activity and the resulting effects on transport of photosynthates from sites of production to sink tissues including roots, flowers, growing tips, and fruits (Fletcher, 2001). Foliar chlorosis, reduction of the sizes of plant organs, stunting and wilting, phloem discoloration and necrosis, and eventual plant death, may result from active or passive spiroplasma roles. Although a given sieve tube cell may harbor few spiroplasmas, others may support as many as 10^{13}/ml (Daniels et al., 1982), a population level that must surely impact normal phloem function as the pathogen usurps nutrients intended for plant functions and may also affect the rate of sugar transport within the phloem. Foliar and stem chlorosis may be caused by the lack of photosynthates, which could result from spiroplasma competition for phloem sugars, or by the secretion of spiroplasma substances that cause chlorophyll degeneration. The major sugar in plant phloem is sucrose, a disaccharide of glucose and fructose. Although each of these monosaccharides can be metabolized by spiroplasmas when present unconjugated, the disaccharide sucrose cannot (Bové et al., 2003). Enzymes for sugar metabolism may play an important role in spiroplasma virulence (see the section Virulence Mechanisms: Sugar Metabolism in this Chapter).

CHEMICAL IMBALANCES: NUTRIENT COMPETITIION, PLANT GROWTH REGULATORS AND INHIBITORS. Plant stunting, internode shortening, and leaf size reduction could result from nutrient reduction caused by competition between spiroplasmas and their host cells for the sugars and other elements present in phloem sap (Chang, 1989). There also is evidence for significant accumulation of lactic acid in spiroplasma growth media (Saglio et al., 1973). If similar accumulation occurs also in planta, it could inhibit normal plant functions. Stubborn-affected citrus trees exhibited higher concentrations of linalool, a leaf oil (Scora et al., 1972), and altered levels of certain organic acids (Bové et al., 1961), but the significance of these findings is unclear.

A number of symptoms caused by spiroplasma infection, including stunting and chlorosis, resemble those caused by an imbalance of host plant growth regulators. Chang, using *S. citri*- or aster yellows phytoplasma (AYP)-infected periwinkle shoot and root tip cultures to examine such interactions, demonstrated that the concentrations of auxin and cytokinin required for maximum shoot proliferation in the two groups differed significantly, indicating that the two mollicutes cause different changes in the endogenous levels of these hormones (Chang, 1989; Chang, 1998). *Spiroplasma citri*-infected tissues also sustained a greater reduction than AYP-

infected ones in the auxins required for optimal root development. Sterols, which are intermediates in many growth regulator synthetic pathways, may be usurped by the invading spiroplasmas, which require sterols for growth. Chang (1998) also investigated levels of pigment production in periwinkle plants infected with either *S. citri* or AYP. A large reduction measured in the levels of the carotenoid pigments in periwinkle plants infected with both mollicutes was consistent with the chlorosis often seen with both diseases, although concentrations of carotenoids in spiroplasma-infected leaves alone were not affected. *Spiroplasma citri* infection also resulted in lowered levels of the red- and purple-hued anthocyanin pigments.

TOXINS. At least one spiroplasma, *S. citri*, causes wilting and death of its plant hosts (citrus, horseradish and periwinkle). These symptoms appear rapidly after the necrosis and degeneration of the plant roots, and are probably due to the failure of the plant to uptake water. The involvement of a spiroplasma toxin has been suggested as the cause of root death, and in fact an *S. citri* toxin was shown to affect the physiology of tobacco leaf discs and a green alga (Daniels et al., 1973), but since the toxin's instability prevented its characterization, the question of whether toxins play a role in natural infections remains unanswered. Another factor related to water availability in mollicute infected plants is stomatal closure (Matteoni and Sinclair, 1983). Measured as the diffusive resistance to water of the leaf surface, stomatal closure was a prominent plant reaction to infection by both phytoplasmas and *S. kunkelii*, suggesting that it is a general symptom of yellows diseases. Further, the degree of closure was proportional to symptom severity. For corn plants infected with *S. kunkelii,* stomatal closure was significantly greater within the white-striped areas of leaves than in the green areas. Cytopathology was also reported in the affected corn leaves; epidermal cells within the white stripes did not elongate normally, most of the guard cells failed to differentiate completely, and infected leaves had more than twice as many stomata per unit area than healthy leaves. The physiological explanation for the stomatal effects is not known, but the authors noted that closed guard cells accumulate abscisic acid, a growth regulator associated with leaf yellowing and early leaf drop. Stomatal closure also would reduce CO_2 uptake, cause a rise in temperature in the affected tissues, and possibly inhibit xylem transport. Other cytopathic effects were noted by Overman et al. (1992), who found that the guard cells in *S. kunkelii*-infected plants had an unusual rounded morphology, and that associated cells fused with the adjacent epidermal cells.

SECRETORY SYSTEMS. Proteins secreted into the external milieu of the spiroplasmas are key candidates for involvement in host-pathogen interactions. As expected, knowledge of the complete genome sequence of *S. kunkelii* has allowed the identification of genes encoding proteins whose signal peptides contain motifs associated with transport functions, leading to the prediction of at least four protein secretion mechanisms, including a Sec-dependent pathway, a type IV secretion system, a twin-arginine mechanism, and an ABC transporter system (Zhao et al., 2004). The latter of these systems contains ATP-binding cassette (ABC) domains in 11 membrane-associated and multicomponent transporters involved in import and export of substances related to nutrition, drug resistance, and possibly virulence. Two other ABC domains occur in cytosolic systems having roles in DNA repair and response to oxidative stress. *Spiroplasma citri* also contains gene systems related to transport and secretion. The anticipated public release of the *S. citri* genome sequence (Foissac et al., 2004) will facilitate searches for additional secretion or signaling pathway genes.

PILI AND FIMBRIAE. As mentioned in the section Morphology, recent electron microscopy studies of the morphology of *S. kunkelii* revealed the presence of long, narrow surface appendages that were interpreted to be fimbriae and pili (Ozbek et al., 2003; Ammar et al., 2004; Bai et al., 2004). Similar surface appendages on other bacteria have been implicated in a number of different functions, some of which (such as recognition and attachment to host surfaces, uptake or delivery of pathogenesis- or defense-related DNA or proteins) may be involved in virulence. On the other hand, the authors (Ammar et al., 2004) noted that the surface structures seen in the micrographs could be spiroplasma viruses. Their size ranges are compatible with the latter explanation.

TRA GENES AND FUNCTIONS. Genes homologous to *traE* genes of other bacteria have recently been reported in both *S. citri* (Foissac et al., 2004; Joshi et al., 2004) and *S. kunkelii* (Bai et al., 2004; Zhou et al., 2004). Zhou et al. (2004) identified a 14.6-kb *S. kunkelii* CR2-3X plasmid, pSKU146, containing *tra*-like genes, as well as a homolog of the *S. citri* adherence-related gene *arp*, first reported by Berg et al. (2001). Joshi et al. (2004) found that the *S. citri* BR3-3X *arp* gene was located on a high copy number plasmid, the 13-kb pBJS. The same plasmid also carried open reading frames homologous to *soj*, a gene associated with chromosome partitioning, and *traE* and *mob*, two genes that, in other bacterial species, have roles in ATP-dependent DNA transfer and mobilization. The *S. citri* gene, *arp2*, a partial homolog of *arp1* that encodes a similar protein,

SARP2, is chromosomally encoded (Joshi et al., 2004). Both *tra* and *soj* genes were predicted from the sequences of six putative plasmids, pSci1–pSci6, of *S. citri* strain GII3 (Foissac et al., 2004). The presence of genes such as *tra*, *mob*, *soj* and *arp* on plasmids may contribute to the acquisition and stability of the plasmid in spiroplasmas, in addition to contributing to their leafhopper transmissibility and plant pathogenicity. Two *tra* genes and *arp*-related genes identified in another *S. kunkelii* strain, M2, were located on a plasmid (Bai et al., 2004). Predicted protein products of the M2-associated *tra* genes had transmembrane regions and ATPase domains.

SUGAR METABOLISM. Although metabolic capabilities are not usually considered virulence factors, aspects of sugar metabolism have been identified as central to the pathogenicity of *S. citri* (Gaurivaud et al., 2000; Gaurivaud et al., 2001; Bové and Garnier, 2002; Bové et al., 2003). Spiroplasmas residing in phloem sieve tubes use glucose and fructose as major sources of energy. When insertion of a transposon into the *S. citri* genome led to a mutant displaying reduced pathogenicity, the interrupted gene was identified as *fru*R, the first gene in an operon involved in fructose utilization. Other genes in the operon include *fru*A, encoding fructose permease, and *fru*K, encoding 1-phosphofructokinase, both of which contribute to the spiriroplasmas' ability to utilize fructose as an energy source. The *fru*R mutants, unable to utilize fructose, induced symptoms much later than wildtype spiroplasmas, and the symptoms were mild. Bové and Garnier (2002), noting that the fructose concentration in sieve tubes is normally low while sucrose concentrations are very high, speculated that sucrose loading by companion cells is fructose-dependent. Utilization of this low-abundance sugar by spiroplasmas would cause impairment of sucrose loading into sieve tubes, resulting in symptoms such as slower plant growth and chlorosis. The fact that *fru*R mutants still induced mild symptoms indicated that fructose metabolism is not the only factor in spiroplasma pathogenicity.

The regulation of the fructose operon also is relevant to the spiroplasma's niche adaptation (Gaurivaud et al., 2000). Expression of *fru*R is upregulated in the presence of fructose, and the FruR protein is an activator of the operon. Interestingly, when spiroplasmas colonize the hemolymph of their insect vectors, where trehalose, rather than glucose and fructose, is the most prevalent sugar, they begin to metabolize the trehalose instead. The organization of the spiroplasma operon for trehalose utilization is very similar to that of the glucose and fructose operons. Andre et al. (2003) characterized the permease II enzymes (EII^{Glc} and EII^{Tre}) of the *S. citri*

phosphoenolpyruvate:glucose and phosphoenolpyruvate:trehalose phosphotransferase systems (PTS). The former contains three domains distributed in two cistrons (A and BC), all necessary for activity. EII^{Tre} lacks the A cistron, whose function is supplied by that of EII^{Glc}. Andre et al. speculated that the ability of the EII^{Glc} A cistron to function with both the glucose and trehalose permeases might be an adaptive strategy, allowing the spiroplasmas to convert their sugar utilization strategies efficiently during the niche changes associated with transmission to a new host (see also the section Physiology and Metabolism in this Chapter).

PHYTOPATHOGEN-UNIQUE SEQUENCES. The availability of the genome sequences of several phytopathogenic mollicutes allowed the recent identification of putative phytopathogenicity-related genes common to three phytopathogens: the aster yellows (AY) phytoplasma, the onion yellows (OY) phytoplasma, and *S. kunkelii* (Bai et al., 2004). These species were not among the obligate animal or human pathogenic mollicutes for which genomic sequences were available. The proteins putatively encoded by these four genes unique to the plant pathogens included a polynucleotide phosphorylase, an exoribonuclease of the PDX family, which, in other bacteria, is involved in mRNA degradation and the regulation of genes associated with invasion and multiplication. In the insect-transmitted mollicutes of plants, the frequent movement between the plant and insect hosts may require such enzymes for rapid adaptation to the alternative niches. A second putative protein has similarity to the C-repeat binding factor (CBF) gene of *Staphylococcus aureus*, which binds to a *cmp* DNA sequence to facilitate plasmid replication. A third, a cytosine deaminase, functions in nucleotide metabolism and protein synthesis. A fourth putative protein resembles YlxR, a protein that binds to RNA and is suggested to be involved in gene expression. This study provides an example of the types of information that will become even more available and useful as the number of complete genome sequences increases.

HOST REACTIONS. The colonization of the very specialized phloem sieve tubes by the phytopathogenic mollicutes in general, and by spiroplasmas in particular, raises the question of whether such infections set off plant defense responses such as the production of reactive oxygen species, polyphenols, phytoalexins, signaling cascades, etc., that are commonly activated following infection by nonvascular pathogens. Also unclear is whether plant pathogenic vascular-limited prokaryotes are detected by plant host sensors in a manner that causes activation of defense pathways. If such defense reactions are

initiated, researchers do not know whether defense-associated molecules are delivered into phloem sieve tubes, or, if they are, whether mollicutes are affected by them. However, maize cultivars resistant to corn stunt spiroplasma are available.

In 2001, Jagoueix-Eveillard et al. examined plant mRNAs that were up- or downregulated following infection of periwinkle with one of three mollicutes, *S. citri*, Candidatus *Phytoplasma aurantifolia*, and the stolbur phytoplasma. Of the 24 differentially expressed cDNAs chosen for study, 8 were similar to known genes encoding proteins related to photosynthesis, sugar transport, stress responses, and sterol biosynthesis. One gene induced in periwinkle by *S. citri*, and known to be induced in *Arabidopsis* by salicylic acid (He et al., 1999), encodes a plant defense-related cell wall-associated kinase. Jagoueix-Eveillard and colleagues reported that the defense reactions observed could not be correlated with particular pathogens but could be associated with the production of particular symptoms.

Control

Our current ability to manage spiroplasma diseases of plants is limited and is best approached with a multi-pronged approach (Fletcher, 2001b).

Traditional Methods

Corn stunt disease, affecting an annual crop, has been managed by several methods including eradication of affected plants, field sanitation, and removal of alternate hosts (*Tripsacum* sp; Tsai and Miller, 1995). In many parts of the world, maize is grown in various intercropped systems, often with beans and sometimes also with cucurbits (cucumbers, melons, pumpkins and squash). Maize diseases caused by insect transmitted viruses may be less severe in their impact in intercropped fields, possibly because of altered crop-insect dynamics related to plant density and diversity. To investigate the hypothesis that the same might be true for maize bushy stunt phytoplasma and the corn stunt spiroplasma, Castro et al. (1992) measured disease incidence and pathogen identity in several maize-bean intercropped field designs (Fig. 8), but no difference in disease incidence was found.

Management of citrus stubborn disease is somewhat more challenging because of the perennial nature of the crop and the high value of individual trees. Recommendations include the location of citrus nurseries in regions having little or no spread of *S. citri*, selection of propa-
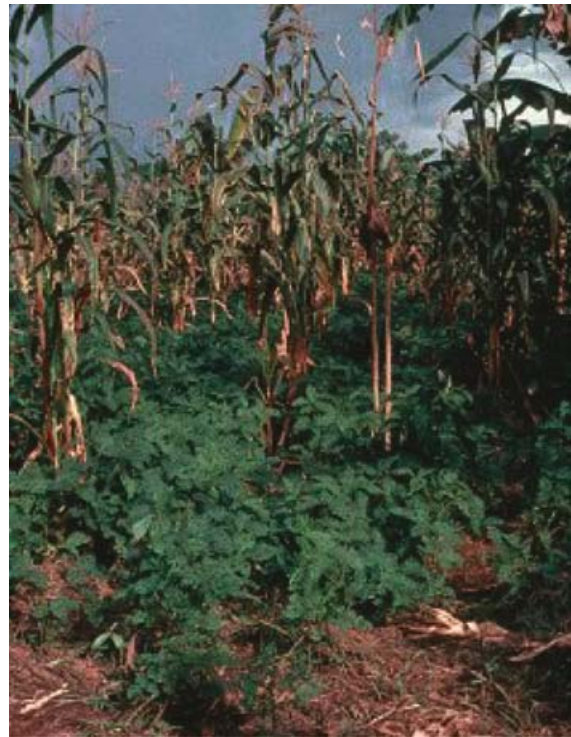
Fig. 8. Maize-soybean intercropping in Costa Rica. Photo: M. E. Irwin, Illinois Natural History Survey, Champaign, IL.

gation scions from healthy trees, elimination of brassicaceous weed hosts from field borders and groves, regular inspection of nurseries and groves, and roguing of infected plants (Fletcher, 2001a).

Because of the sporadic nature of horseradish brittle root disease, little is known about the effectiveness of cultural methods to control the disease. Since the vector *Circulifer tenellus* thrives best in hot, dry, exposed field areas, irrigation of affected fields may reduce drought stress on plants and make the fields less hospitable for beet leafhopper reproduction and development. After a brittle root outbreak, horseradish growers should take care to select healthy root cuttings for planting the following year to minimize carryover of the spiroplasma. Roguing plants that become symptomatic early in the season (likely to have grown from cryptically infected root cuttings) may minimize infield spread.

Host Resistance

In some cases, germplasm encoding natural resistance to spiroplasmas or their vectors is available, and such genes are incorporated into breeding programs for high-value crops, vegetables and fruits, and ornamentals (Fletcher, 1999). Additional resistance genes may be found in wild
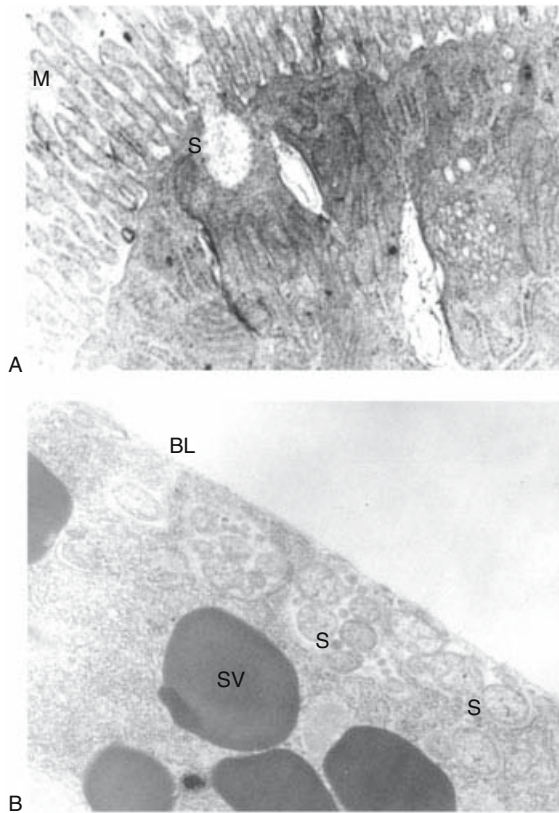
Fig. 9. A) Section through the midgut of *S. citri*-exposed *C. tenellus* showing a spiroplasma cell apparently fixed during its invasion of a midgut epithelial cell. S, spiroplasma cell; M, epithelial microvillae. B) Spiroplasmas in membrane-bound pockets within the salivary gland of *C. tenellus*. S, spiroplasma cell; SV, secretory vesicle; BL, basal lamina. Photos: M. Kwon and A. Wayadande.

Fig. 10. Corn plant showing symptoms of corn stunt disease: stunting, foliar chlorosis, and reddened streaking. Photo: M. E. Irwin, Illinois Natural History Survey, Champaign, IL.

progenitors of crop species or in other plants. Plant defense chemicals are known to be involved in host defense reactions against other pathogens, but whether such chemicals are effective against mollicutes, and whether they are delivered to phloem sieve tubes, remain unanswered questions. Only a few investigators have explored the differential expression of plant genes in the presence of mollicutes. See the section Virulence Mechanisms in this Chapter.

Chemical Treatments

Antibiotic treatments have been of some usefulness for management of "yellows" diseases in non-food plants of high value, such as ornamentals (Sinha, 1979; McCoy and Williams, 1982). The efficacy of certain antibiotics against spiroplasmas depends on their pore forming ability (Beven et al., 1999). The methyl ester of amphotericin B was effective against *S. citri* in culture (Goldstein et al., 1976). Penicillins and other antibiotics that interfere with bacterial cell wall

synthesis have no effect on the wall-less mollicutes. Antibiotics in the tetracycline family, with a mode of action related to interference with protein and nucleic acid synthesis, are most effective. Chloramphenicol was somewhat less effective when tested for control of phytoplasma diseases. Treatment often leads to temporary remission of symptoms, an effect that persists for varying periods depending on the method of application, which may include spraying, root or shoot dips, or vascular infusion. Tetracycline is translocated in the xylem, becoming systemically distributed and apparently ultimately being uploaded into the phloem. Unfortunately, symptoms reappear soon after suspension of treatment, and antibiotic treatments have the added concern of residues in plants used for food and feed (Sinha, 1979; Fletcher, 2001). Tetracyclines have not proven beneficial in citrus (Fletcher, 2001a). Interestingly, streptomycins are effective against *S. citri* in culture but not in planta (McCoy and Williams, 1982). Although this antibiotic moves through the plant in the xylem, it apparently is not phloem-mobile. Few examples of successful management of spiroplasma diseases by chemicals other than antibiotics exist,

but quinine and quinoline compounds protected fruit trees against another mollicute, the X-disease phytoplasma (Sinha, 1979).

Insect Management

Managing spiroplasma vectors through the use of insecticides is challenging because the insects must be killed before they feed on host plants. However, epidemiological considerations may make insect control effective under some conditions. In California citrus production areas, the elimination of potential vector species in weedy areas prior to weed removal will prevent insect migration into adjacent citrus groves (Oldfield, 1984). Extensive use of insecticides may have reduced the incidence of horseradish brittle root disease in epidemic years, possibly by reducing secondary spread from brittle root affected to healthy plants (Fletcher et al., 1981). Recently, when investigating a significant incidence of corn stunt disease sustained in silage corn production in the southern San Joaquin Valley, California, Frate et al. (2004) found that seed treatments with the systemic insecticides (imidacloprid and clothianidin) significantly reduced both the numbers of vector leafhoppers, *D. maidis*, and the incidence of stunt disease.

Plantibodies

The fact that monoclonal and polyclonal anti-spiroplasma antibodies inhibit the pathogens' growth and metabolism in vitro led investigators to hypothesize that transgenic plants, engineered to express such antibodies, would be disease resistant. Noting that recombinant single-chain variable fragment (scFv) antibodies were excellent candidates for expression in plants, Chen and Chen (1998) demonstrated that scFv antibodies prepared against *S. kunkelii* were expressed in the cytoplasm of tissue cultured maize cells. However, although the cell line showed spiroplasma-binding activity, plants regenerated from the transgenic cells were indistinguishable from wild-type plants in their susceptibility to the spiroplasma. The authors speculated that the lack of resistance could have resulted from a lower inhibitory activity of the scFv antibodies compared to polyclonal or monoclonal antibodies, a lack of antibody expression at the actual spiroplasma colonization site (plant sieve tubes), or a lower level of antibody expression in the regenerated plants than in the transgenic cell line. Later, Malembic et al. (2002) demonstrated that scFv antibodies against *S. citri* were able to inhibit spiroplasma growth and motility, although significant spiroplasma growth inhibition might require a multiplexed system involving expression of two or more three such antibodies in combination. These authors also broached the possibility of delivering antibodies to plant sieve tubes using engineered, nonpathogenic spiroplasma strains.

Future Possibilities

Although our ability to manage plant diseases caused by spiroplasmas is currently very limited, significant advances in our understanding of how spiroplasmas cause plant malfunctions and interact with their insect vectors will create new possibilities for maintaining healthy plants despite the threat of spiroplasma diseases.

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CHAPTER 1.3.1

The Family Heliobacteriaceae

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Introduction

The family Heliobacteriaceae consists of phototrophic bacteria that contain bacteriochlorophyll (Bchl) *g* (Fig. 1). This unique Bchl, found only in the heliobacteria, distinguishes them from all other anoxygenic phototrophic bacteria (Madigan, 2001; Madigan and Ormerod, 1995b). The heliobacteria were discovered in a very serendipitous fashion by Howard Gest and Jeff Favinger in the early 1980s; a fascinating account of this discovery can be found in Gest (1994). The unique characteristics of heliobacteria were first revealed a few years later with publication of the description of *Heliobacterium chlorum* (Gest and Favinger, 1983) and the structure of Bchl *g* (Brockmann and Lipinski, 1983). Subsequent research in the Gest laboratory led to the discovery of *Heliobacillus*, and with this, the establishment of the family Heliobacteriaceae (Beer-Romero and Gest, 1987). Four genera of heliobacteria are currently recognized: *Heliobacterium*, *Heliobacillus*, *Heliophilum* and *Heliorestis* (Madigan, 2001).

Besides producing Bchl *g*, heliobacteria can also be distinguished from all other anoxygenic phototrophs by their lack of differentiated photosynthetic internal membranes, such as the membrane vesicles or lamellae of purple bacteria or the chlorosomes of green bacteria. As far as has been determined, photosynthetic complexes in heliobacteria reside in the cytoplasmic membrane (Miller et al., 1986). Heliobacteria are also phylogenetically distinct anoxygenic phototrophs. They group with the low G+C Gram-positive bacteria; specifically, with endosporulating bacteria such as *Clostridium* species (Madigan, 2001; Woese et al., 1985).

Bacteriochlorophyll *g* shows structural relationships with both chlorophyll *a* and with the various bacteriochlorophylls of green and purple bacteria (Brockmann and Lipinski, 1983; Michalski et al., 1987). Like green plant chlorophyll *a* but unlike all bacteriochlorophylls, Bchl *g* contains a vinyl ($H_2C=CH_2$) group on ring I of the porphyrin tetrapyrrole. However, like bacteriochlorophylls *a* and *b* and unlike chlorophyll *a*,

pyrrole ring II of Bchl *g* is reduced (Brockmann and Lipinski, 1983; Fig. 1). Moreover, the esterifying alcohol of Bchl *g* is farnesol (Michalski et al., 1987), the alcohol that also esterifies the bacteriochlorophylls of green sulfur bacteria (Gloe et al., 1975), instead of the phytol or geranylgeraniol found in Bchls *a* and *b* or the phytol of green plant chlorophylls (Oelze, 1985).

The novel bacteriochlorophyll of heliobacteria is responsible for the unique absorption properties of these phototrophs. Heliobacteria absorb maximally in the near infrared at 788 nm (Fig. 2), distinctly away from regions of the spectrum absorbed by green bacteria and purple bacteria; the latter groups typically show near infrared absorption maxima at 705–740 nm or 830–1100 nm, respectively (Oelze, 1985). The absorption maximum of Bchl *g* represents a "window" in the spectrum of all other phototrophic organisms (Madigan, 1988; Pfennig, 1989) and this characteristic undoubtedly contributes to the ecological success of the heliobacteria in nature.

The photosynthetic reaction center of heliobacteria is contained along with associated antenna pigments in a single pigment-protein complex (Amesz, 1995). And, although the primary photosynthetic electron donor in heliobacteria is Bchl *g*, the primary electron acceptor is a form of chlorophyll (not bacteriochlorophyll) *a* called hydroxychlorophyll *a* (Amesz, 1995). This and other molecular features of heliobacterial photocomplexes (Amesz, 1995) highlight the structural (and thus phylogenetic) relationship between them and green plant photosystem I (Amesz, 1995; Gupta et al., 1999; Schubert et al., 1998; Stackebrandt et al., 1996). Molecular comparisons of pigment biosynthesis genes further support a close phylogenetic connection between the heliobacteria and oxygenic phototrophs (Xiong et al., 2000).

Besides its unique Bchl, heliobacteria also synthesize novel carotenoids. Although heliobacteria were originally thought to contain only a single carotenoid (the C_{40} pigment neurosporene; Van Dorssen et al., 1985), a reexamination of the carotenoids of several species of heliobacteria has shown that there are mainly
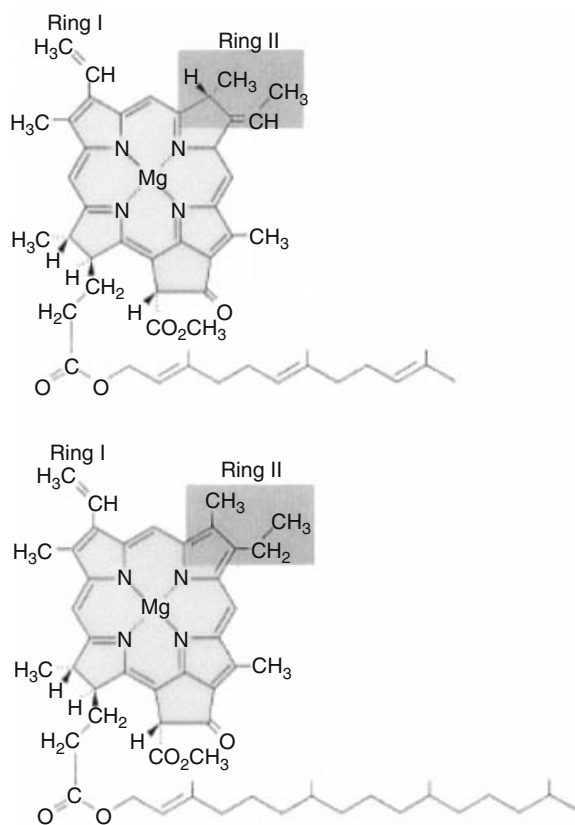
Fig. 1. Structures of bacteriochlorophyll *g* (top) and chlorophyll *a* (bottom). Note that both pigments are structurally identical on rings I and II except that ring II (shaded in both structures) is reduced in bacteriochlorophyll *g* (Bchl *g*). Also note that the esterifying alcohols are farnesol in Bchl *g* and phytol in chlorophyll *a*.
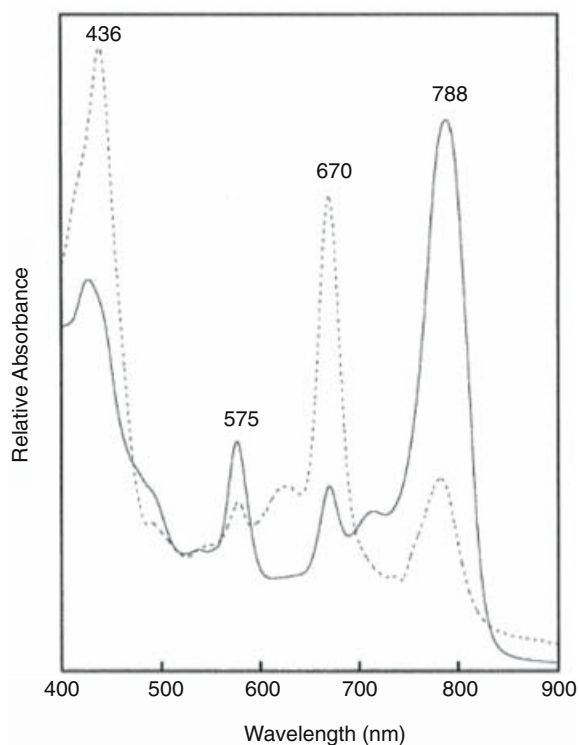
Fig. 2. Absorption spectrum of intact cells of the thermophilic heliobacterium, *Heliobacterium modesticaldum*. Solid line, cells suspended in 30% bovine serum albumin that was reduced by adding 0.05% ascorbate. Dashed line, spectrum of the same cell suspension exposed to light and air for one hour. Note major peak of bacteriochlorophyll *g* at 788 nm and of chlorophyll *a* at 670 nm (L. K. Kimble and M. T. Madigan, unpublished observations).
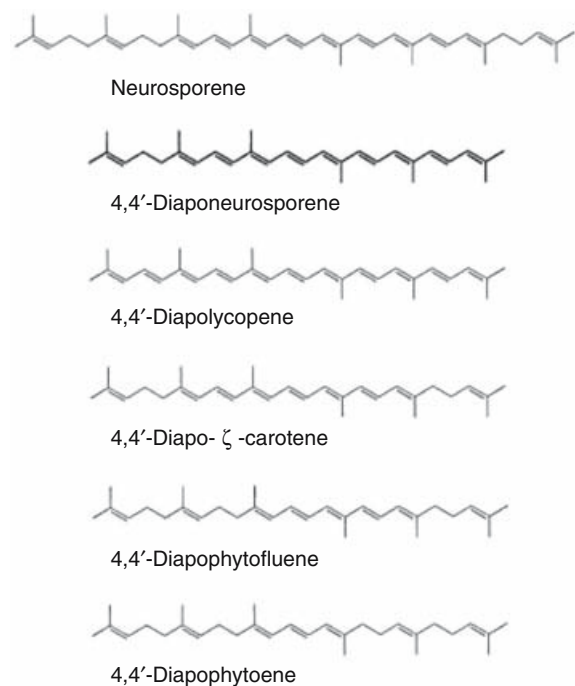
five structurally related C_{30} carotenoids spectrally resembling neurosporene and only traces of neurosporene itself; the pigment 4,4'-diaponeurosporene predominates in all species examined (Takaichi et al., 1997; Fig. 3). The synthesis of C_{30} carotenoids is another feature that makes heliobacteria unique from a photosynthetic perspective; carotenoids in all other phototrophic organisms are C_{40} derivatives (Takaichi, 1999; Takaichi et al., 1997).

Fig. 3. Carotenoids of heliobacteria. Neurosporene (C_{40}) is present in only trace amounts in heliobacteria while the remaining pigments (all C_{30} carotenoids) are present in larger amounts. Of the five C_{30} pigments shown, 4, 4′diaponeurosporene (shown in bold) was the dominant carotenoid found in five species of heliobacteria examined. (From Takaichi et al., 1997, with permission.)

Phylogeny

The phylogeny of heliobacteria (as deduced from comparative 16S ribosomal RNA sequencing) places them within the low G+C Gram-positive bacteria (Woese, 1987), specifically within the clade containing the endosporulating rod-shaped bacteria *Bacillus* and *Clostridium* (Madigan, 2001; Woese et al., 1985). The four known genera of heliobacteria (Table 1) form a rather tight cluster near the reductive dechlorinating organism *Desulfitobacterium dehalogenens* (Fig. 4). Lying basal to all known heliobacteria is *Heliorestis daurensis*, a morphologically and physiologically unique species (Bryantseva et al., 1999; see Habitat; Fig. 4).

Although the basis for the phylogenetic connection between phototrophic heliobacteria and nonphototrophic endosporulating anaerobes remains obscure, the grouping of the heliobacteria with the latter, first proposed in 1985 by Woese et al. (1985), was indeed prophetic. As might have been predicted from their phylogenetic position, heliobacteria were later discovered to produce endospores (see Taxonomy); like those of *Bacillus* and *Clostridium* species, heliobacterial endospores were shown to contain elevated levels of Ca^{2+} and dipicolinic acid (Ormerod et al., 1990; Ormerod et al., 1996).

The Gram-positive connection between heliobacteria and clostridia is enigmatic, as it concerns cell wall structure and chemistry. Unlike typical clostridia, heliobacteria contain rather thin cell walls and actually stain Gram-negatively; however, no evidence for a lipopolysaccharide (LPS)

outer membrane has been obtained (Beer-Romero et al., 1988; Madigan, 2001). By contrast, several truly Gram-positive features of heliobacteria cell walls are apparent and include peptidoglycan containing L, L- diaminopimelic acid (instead of *meso*-diaminopimelic acid) in muramic acid cross-links, and the presence of a glycine interbridge (Beer-Romero et al., 1988; Pickett et al., 1994).

Fatty acid and cytochrome analyses of heliobacteria have also strengthened their Gram-positive connection. Heliobacteria contain a high proportion of branched-chain fatty acids, a common feature of Gram-positive bacteria, with up to 60% of the fatty acids in various species being branched-chained; of the latter, isopalmitoleic acid typically predominates (Beck et al., 1980). Moreover, molecular analyses of the dominant *c*-type cytochrome in heliobacteria, cytochrome c_{553}, have shown it to be closely related to the homologous protein from *Bacillus* species (Albert et al., 1998).

In summary, grouping of the heliobacteria with other low G+C Gram-positive bacteria is supported by 1) comparative ribosomal RNA sequencing, 2) the production of endospores, 3) certain but not all aspects of cell wall chemistry, 4) fatty acid composition, and 5) cytochrome *c* type. Other connections between heliobacteria and Gram-positive bacteria, especially the clostridia, will become apparent in the sections on Habitat and Physiology.

Taxonomy

Four genera with a total of seven species are currently officially recognized within the family Heliobacteriaceae (Madigan, 2001). The genus *Heliobacterium* and the species *H. chlorum* (Fig. 5) was the first of the heliobacteria to be discovered, but for various reasons, including the tendency for cells of this species to lyse when reaching late exponential phase (Gest and Favinger, 1983), it is not the major species worked with today. The genus *Heliophilum* is unusual because its cells form bundles that move as a unit (Fig. 6).

Most species of heliobacteria are rod-shaped, typically with tapered cell ends (Fig. 7), and are motile by flagellar means (Fig. 8). Cells of *Heliobacterium gestii* are spiral-shaped (Bryantseva et al., 1999). Cells of heliobacteria show no remarkable fine structure, as no differentiated photosynthetic membrane systems of any type are apparent (Fig. 9). All heliobacteria have genus names that begin with "Helio," a combining form from the Greek word "helios," meaning "sun." A summary of the major properties of described species of heliobacteria is presented in Table 1.
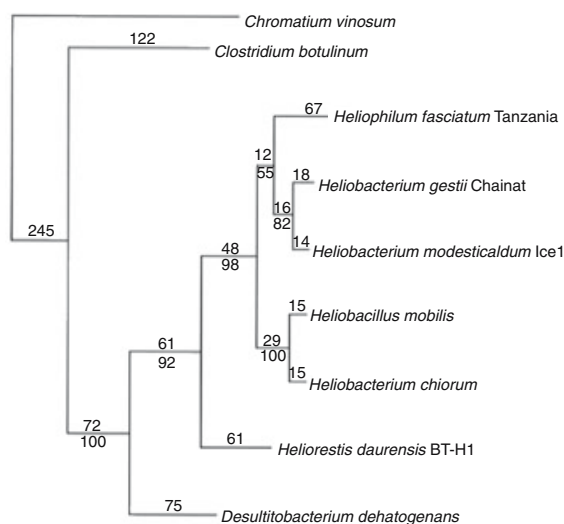
Fig. 4. Phylogenetic tree of heliobacteria based on comparative 16S rRNA sequences and analysis by parsimony. (From Bryantseva et al., 1999, with permission.)

Table 1. Summary of properties of heliobacteria.

Property	Heliobacterium chlorum	Heliobacterium gestii	Heliobacterium modesticaldum	Heliobacillus mobilis	Heliophilum fasciatum	Heliorestis daurensis	Heliorestis baculata
Habitat	Temperate soil	Tropical paddy soil	Neutral/alkaline hot springs and volcanic soils	Tropical paddy soil	Tropical paddy soil	Microbial mats of brackish soda lakes	Soda lake shoreline soil
Morphology	Rod	Spirillum	Rod/curved rod	Rod	Straight rods with tapered ends grouped in bundles of two to many cells	Coiled to bent rod or short filament	Rod
Dimensions	1×7–$9\mu m^a$	1×7–$10\mu m^b$	0.8–1×2.5–$9\mu m^c$	1×7–$10\mu m$	0.8–$1\mu m \times 5$–$8\mu m$	0.8–$1.2\mu m \times 10$–$20\mu m$	0.6–$1\mu m \times 6$–$10\mu m$
Motility	Gliding	Multiple subpolar flagella	Flagellar or none	Peritrichous flagella	Polar to sub-polar flagella; cell bundles move as a unit	Peritrichous flagella	Peritrichous flagella
Carbon sources photo-metabolized	Pyruvate, lactate and yeast extract	Pyruvate, lactate, acetate, butyrate, ethanol ($+CO_2$), yeast extract, glucose and fructose	Pyruvate, lactate, acetate and yeast extract	Pyruvate, lactate, acetate, butyrate ($+CO_2$) and yeast extract	Pyruvate, lactate, acetate and butyrate ($+CO_2$)	Pyruvate, acetate and yeast extract	Pyruvate, acetate and yeast extract
Biosynthetic sulfur sources	SO_4^{2-}, $S_2O_3^{2-}$, methionine, or cysteine	$S_2O_3^{2-}$, methionine, or cysteine	$S_2O_3^{2-}$, sulfide, methionine, or cysteine	SO_4^{2-}, $S_2O_3^{2-}$, methionine, or cysteine	SO_4^{2-}, $S_2O_3^{2-}$	Sulfide	Sulfide
Endospores[d]	None observed	Yes	Yes	None observed	Yes	Yes	Yes
Optimum temperature, °C	37–42	37–42	50–52	38–42	37–40	30	30
Optimum pH	7	7	6–7	6.5–7	6.5–7	9	8.5–9.5
Mol% G+C content	52	55.1	54.6–55	50.3	51.8	44.9	45

[a]See Fig. 2.
[b]See Fig. 3.
[c]See Fig. 4.
[d]Described species of heliobacteria produce endospores sporadically (if at all) in pure culture.
Data taken from Gest and Favinger, 1983; Beer-Romero and Gest, 1987; Kimble et al., 1995; Ormerod et al., 1996a; Stevenson et al., 1997; and Bryantseva et al., 1999a; 2000.
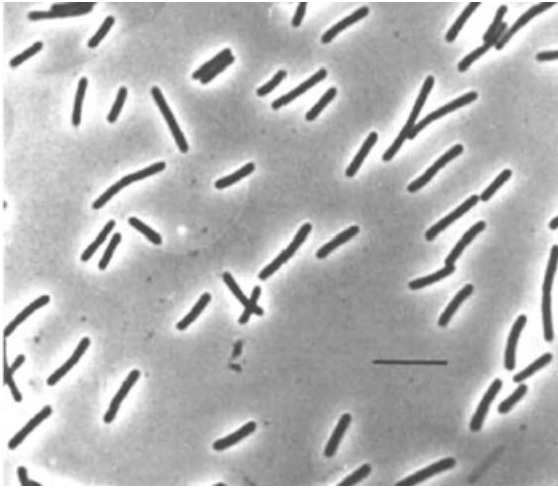
Fig. 5. Phase-contrast micrograph of cells of *Heliobacterium chlorum*. Marker bar, 10 μm. (Courtesy of J. L. Favinger and H. Gest.)
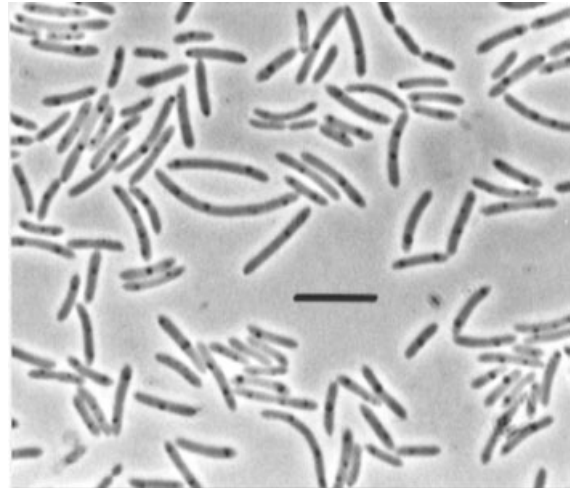
Fig. 7. Phase-contrast micrograph of cells of the thermophilic heliobacterium, *Heliobacterium modesticaldum* strain Ice1. The nature of the presumed storage material (dark spots in cells) is unknown; it is not poly-β-hydroxybutyrate. Marker bar, 3 μm.
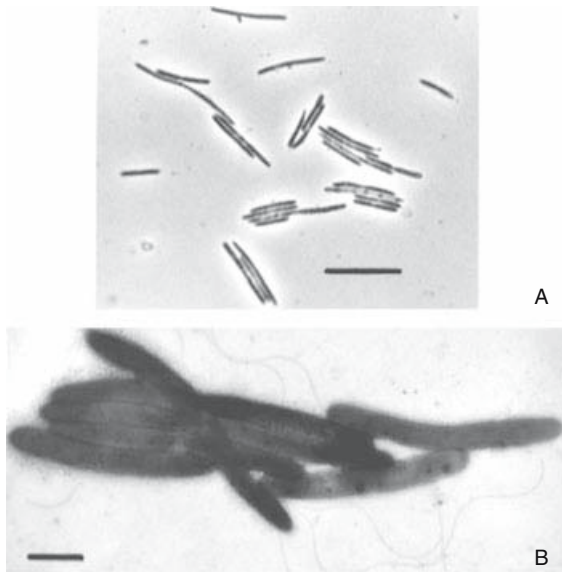
Fig. 6. *Heliophilum fasciatum*. A) Phase-contrast micrograph. B) Negatively stained transmission electron micrograph. Note in both micrographs how cells form into bundles; these bundles are motile as a unit. Marker bars: A, 15 μm; B, 2 μm.
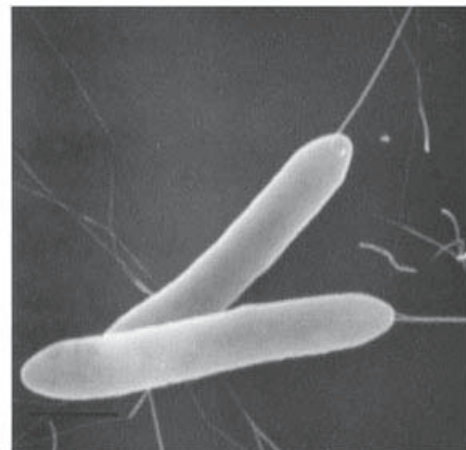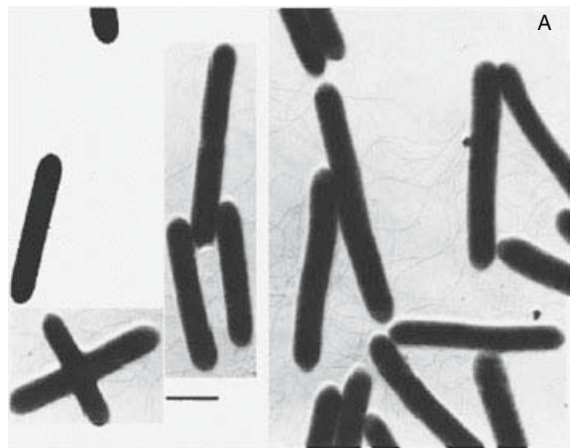
Fig. 8. Flagella in motile heliobacteria. A) Transmission electron micrograph of shadowed cells of *Heliobacillus mobilis*, showing peritrichous flagella. B) Scanning electron micrograph of cells of *Heliobacterium modesticaldum* strain YS5 showing polar/subpolar flagella. Marker bars: A, 2 μm; B, 1 μm.
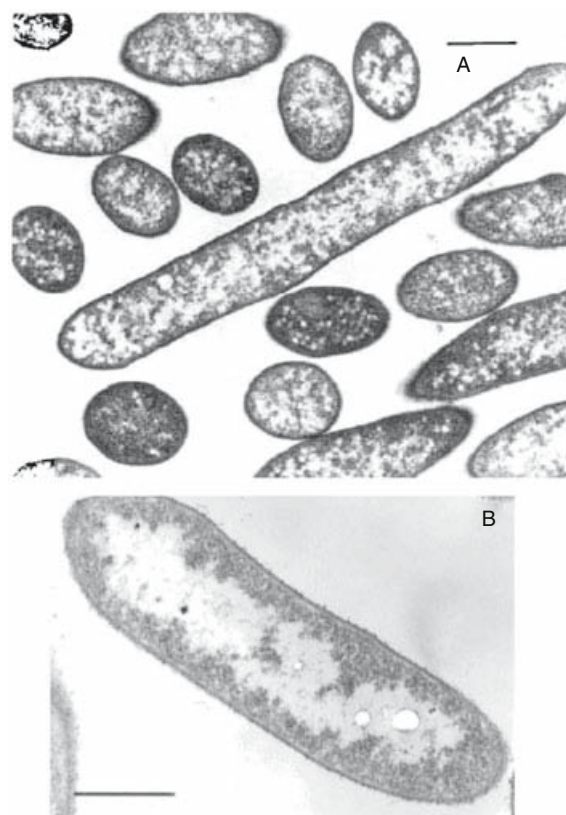
Fig. 9. Transmission electron micrographs of cells of helio-bacteria. A) Cross section of cells of Heliobacterium chlo-rum. Note the studded appearance of the cell surface. (Courtesy of F. Rudi Turner.) B) Longitudinal section of a cell of Heliobacterium modesticaldum strain Ice1. Note absence of intracytoplasmic membranes. Marker bars: A, 0.25 µm; B, 1 µm.

Habitat

Since heliobacteria were first discovered, considerable evidence has accumulated to suggest that soil, in particular rice (paddy) soil, is their major habitat. This goes back to the original isolation of *Heliobacterium chlorum* from garden soil (Gest and Favinger, 1983), and to the subsequent isolations of *Heliobacillus mobilis* (Beer-Romero and Gest, 1987) and *Heliobacterium gestii* and *Heliophilum fasciatum* (Ormerod et al., 1996) from paddy soils.

In a study of potential heliobacterial habitats, 29 of 159 soil, mud or water samples were found to be positive for heliobacteria by enrichment culture assay, and 13 of these enrichments yielded pure cultures (Stevenson et al., 1997). By contrast, none of the water or sediment samples, including both freshwater and marine sources, contained culturable heliobacteria. Notably, many of the driest soil samples tested were the most successful in enrichment cultures for helio-bacteria (Stevenson et al., 1997). This result is likely due to recovery of heliobacterial

endospores rather than to the presence of actual vegetative cells of heliobacteria in these extremely dry soils.

Although the study of Stevenson et al. (1997) did not exhaustively test aquatic habitats for heliobacteria, the fact that none of the aquatic samples tested (except for hot spring waters, see following paragraph) yielded these organisms, suggests that unlike green and purple bacteria (Madigan, 1988; Pfennig, 1989), heliobacteria are not common (or are perhaps even absent) in lakes, ponds and other aquatic environments. The caveat is, of course, that this conclusion is based on data obtained solely from successful or unsuccessful enrichment cultures. Thus the range of habitats of the heliobacteria is still an open question and needs input from other methods such as environmental sampling with specific nucleic acid probes. For example, although everything points to soil as the habitat of helio-bacteria, it is possible that physiologically distinct heliobacteria exist in aquatic habitats whose enrichment requires special conditions not yet understood.

The only exception to the finding that helio-bacteria are soil residents is the discovery of extremophilic heliobacteria in submersed microbial mats. The thermophilic species *Heliobacterium modesticaldum* is widespread in neutral to alkaline hot spring microbial mats (Kimble et al., 1995; Stevenson et al., 1997). Pure cultures of several strains of *H. modesticaldum* grow up to 57°C (optimum 52°C; Kimble et al., 1995) and positive enrichments for this organism were obtained from samples taken at temperatures as high as 70°C (Stevenson et al., 1997).

The alkaliphilic heliobacteria *Heliorestis daurensis* (Bryantseva et al., 1999) and *Heliorestis baculata* (Bryantseva et al., 2000) were also isolated from microbial mats, in this case from mats and soil along the shores of Lake Barun Torey, a soda lake located in the South Chita region of Siberia (Russia). This lake is unusual because of its alkalinity (~pH 9) and low salinity. *Heliorestis daurensis* could be enriched from both cyanobacterial mats surrounding the lake and from soils of dried lagoons adjacent to the lake. Subsequent isolation of *H. baculata*, an alkaliphilic heliobacterium phylogenetically related to *H. daurensis*, was made from soda lake soil near to the mats (Bryantseva et al., 2000). The discoveries of *H. modesticaldum* and the soda lake heliobacteria thus leave open the possibility that freshwater or marine microbial mats also could be habitats for heliobacteria.

Isolation

Enrichment and isolation of heliobacteria begins with an appropriate inoculum. As described in

the Habitat section, soils (especially paddy soils) are good sources of heliobacteria. Indeed, even very dry paddy soil can contain culturable heliobacteria (Stevenson et al., 1997). A variety of media, both defined and complex, have been successful for the enrichment of heliobacteria. But in most cases an additional step in the enrichment process, pasteurization (80°C, 15 min) of the inoculum, has been the key to successful enrichments of heliobacteria (Stevenson et al., 1997). Presumably, heating kills vegetative cells of competing organisms leaving heliobacterial spores to germinate in the semiselective media.

Because heliobacteria are rather strict anaerobes, all media used for their isolation must first be rendered anoxic, such as by boiling the medium for 5 min under a stream of nitrogen and carbon dioxide ($N_2:CO_2$ [95:5]) and then transferring 10-ml aliquots to 25-ml anoxic tubes and sealing under anoxic gas with rubber stoppers. The tubes are then secured in a tube press (Bellco®) and autoclaved for 20 min. Enrichment cultures are established by adding 0.25–0.5 g of sample to 10 ml of anoxic enrichment medium and then vigorously sparging the headspace with $N_2:CO_2$ (95:5) for 30 s and sealing (enrichments to be pasteurized need to have their stoppers wired down before heating). Enrichments should be incubated at 30–40°C (except for thermophilic enrichments, incubated at 48–52°C) and a light intensity of 40–60 $\mu g \cdot m^{-2} \cdot sec^{-1}$ incandescent illumination.

Positive enrichments for heliobacteria are signaled by a green color and spectral evidence for Bchl *g* (Figs. 1 and 2). Liquid from such an enrichment should then be transferred to plates of 0.25% (w/v) yeast extract or pyruvate mineral salts (PMS) medium or pyruvate yeast extract (PYE) medium within an anoxic chamber and streaked for isolation with sterile disposable inoculating loops. Streaked plates can then be incubated in anoxic jars and incubated photosynthetically at the appropriate temperature. For further details on procedures for the enrichment and isolation of heliobacteria, the interested reader should consult the paper by Stevenson et al. (1997).

Culture Media

Enrichments for heliobacteria can be established in a 0.25% (w/v) yeast extract liquid medium or in various defined or even complex media. Pure cultures (except for the alkaliphiles *H. daurensis* and *H. baculata*) typically grow well in medium PMS or PYE. These media can also be used for enrichment and isolation of heliobacteria but have the disadvantage that they also support good growth of a variety of nonphototrophic fermentative bacteria as well.

For most species of heliobacteria that have been isolated thus far, warm (~40°C) enrichment temperatures and high light intensities seem to favor their development and help to select against nonsulfur purple bacteria; the latter can be major competitors in heliobacterial enrichments if moist soil/mud or sediments are used as inocula. Because most heliobacteria also fix N_2 (Kimble and Madigan, 1992a; Kimble and Madigan, 1992b; Stevenson et al., 1997), deleting the ammonia from medium PMS (or its variations) makes it more selective for heliobacteria, especially if the pasteurization step is also employed. Medium PYE is a rich medium that supports luxuriant growth of most pure cultures of heliobacteria, but it is not particularly successful in primary enrichments.

Media Recipes

The following (not commercially available) formulations are mentioned in this chapter.

Pyruvate Mineral Salts (PMS) Medium

The following ingredients are added to 1 liter of double-distilled water:

EDTA	10 mg
$MgSO_4 \cdot 7H_2O$	200 mg
$CaCl_2 \cdot 2H_2O$	75 mg
NH_4Cl	1 g
K_2HPO_4	0.9 g
KH_2PO_4	0.6 g
Sodium pyruvate	2.2 g
Trace element solution (see below)	1 ml
Biotin	15 μg
Vitamin B_{12}	20 μg

Adjust the pH to 6.8 with NaOH or HCl and sterilize by autoclaving for 20 min. After cooling briefly, add either methionine, cysteine or thiosulfate to a final concentration of 1 mM and transfer to an anoxic chamber until used. If thiosulfate is used, it can be added to the medium prior to autoclaving.

Modified PMS Media

1) LMS medium is pyruvate-free PMS medium containing sodium lactate at a final concentration of 20 mM.

2) AMS medium is pyruvate-free PMS medium containing 20 mM sodium acetate plus 0.1% (w/v) $NaHCO_3$.

3) PMS-N medium is PMS medium minus NH_4Cl. The gas in the headspace of the enrichment should be $N_2:CO_2$ (95:5). This medium is suitable for enrichments using nitrogen fixation as a selective tool.

Mixed Acid Mineral Salts (MAMS) Medium

This semi-defined enrichment medium avoids the use of pyruvate and contains three less easily fermented organic acids. The following are added to 1 liter of distilled water:

EDTA	10 mg
NaCl	0.4 g
$MgSO_4 \cdot 7H_2O$	200 mg

CaCl$_2$ · 2H$_2$O	75 mg
NH$_4$Cl	0.8 g
K$_2$HPO$_4$	0.45 g
KH$_2$PO$_4$	0.3 g
NaHCO$_3$	1 g
Trace elements solution (see below)	1 ml
Yeast extract	0.2 g
Vitamin B$_{12}$	20 µg
Sodium acetate	1 g
Sodium malate	2 g
Sodium lactate	1 g

Adjust the pH to 6.8 and sterilize by autoclaving for 20 min. Add (after cooling) NaHCO$_3$ from a filter-sterilized stock solution and either methionine, cysteine or thiosulfate to a final concentration of 1 mM and transfer to an anoxic chamber until used. If thiosulfate is used, it can be added to the medium prior to autoclaving.

Modified MAMS (MAMS-N) Medium MAMS medium minus NH$_4$Cl (MAMS-N) is suitable for heliobacteria and other anoxygenic phototroph enrichments using N$_2$-fixation as a selective tool (Gest et al., 1985). The headspace of enrichment should be filled with N$_2$:CO$_2$ (95:5).

Pyruvate-Yeast Extract (PYE) Medium

This medium is suitable for growth of pure cultures of all species of heliobacteria except those of *Heliorestis*. The following are added to 1 liter of double-distilled water.

K$_2$HPO$_4$	1 g
MgSO$_4$ · 7H$_2$O	200 mg
CaCl$_2$ · 2H$_2$O	20 mg
Na$_2$S$_2$O$_3$ · 5H$_2$O	100 mg
Sodium pyruvate	2.2 g
Yeast extract	4 g

Adjust to pH 7, autoclave, and store in sealed tubes or bottles in an anoxic chamber until used. Alternatively, 1–2 mM sulfide can be added (final pH 6.8) and media dispensed to sterile and completely filled screw-cap tubes or bottles.

Medium Hr for Growth of *Heliorestis daurensis* and *Heliorestis baculata*

For enrichment and culture of alkaliphilic heliobacteria, a modification (designated medium Hr) of the medium described by Bryantseva et al. (1999) is appropriate.

Distilled water	0.6 liter
EDTA	5 mg
Trace elements	1 ml
KH$_2$PO$_4$	0.5 g
NH$_4$Cl	0.5 g
MgSO$_4$ · 7H$_2$O	0.2 g
CaCl$_2$ · 7H$_2$O	75 mg
Na$_2$CO$_3$	2.5 g
NaHCO$_3$	2.5 g
Na acetate	1 g
Yeast extract	0.1 g
Na$_2$S · 9H$_2$O	0.5 g

The carbonate and bicarbonate are autoclaved dry in a 0.5–1-liter bottle and dissolved in 300 ml of sterile distilled water after autoclaving; this solution is then added to the main nutrient solution with gentle stirring. Sulfide is prepared as a separate solution (100 ml) and also added to the main solution after autoclaving. The main salts solution is prepared in a 2–10-liter dispensing jar containing a spigot for dispensing media into tubes or bottles. After autoclaving, all solutions are mixed and the pH is adjusted with sterile HCl or NaOH to 9–9.5; the final assembled medium is immediately dispensed to completely fill screw-cap tubes or bottles and then capped tightly. A slight grayish-black precipitate forms in the medium.

Trace Element Solution for All Heliobacteria Media

The following are added to 1 liter of distilled water:

EDTA (sodium salt)	5.2 g
FeCl$_2$ · 4H$_2$O	1.5 g
ZnCl$_2$	70 mg
MnCl$_2$ · 4H$_2$O	100 mg
H$_3$BO$_3$	6 mg
CoCl$_2$ · 6H$_2$O	190 mg
CuCl$_2$ · 2H$_2$O	17 mg
NiCl$_2$ · 6H$_2$O	25 mg
Na$_2$MoO$_4$·2H$_2$O	188 mg
VSO$_4$ · 2H$_2$O	30 mg
NaWO$_4$ · 2H$_2$O	2 mg

Add compounds in the above order; ethylene diamine tetraacetic acid (EDTA) should be fully dissolved before adding the other components. Store at 4°C.

Growth Vessels

Growth of pure cultures of heliobacteria can be accomplished in either neoprene rubber-stoppered culture tubes (for example, 18 × 142 mm Bellco® anoxic culture tubes), or in screw-cap tubes or bottles; if sulfide is not used, more reliable growth is achieved in stoppered tubes. Screw-cap tubes or bottles should be filled completely and tightly capped. However, because the seal of screw-cap vessels is rarely perfect, growth of heliobacteria requires the addition of 0.05% (w/v; final concentration) of sodium ascorbate. Alternatively, 1–2 mM (final concentration) of sulfide is a good reductant and has not been found growth inhibitory with most species. The best method of media preparation using rubber-stoppered tubes is to add boiled nonsterile media to anoxic culture tubes while constantly degassing with an anoxic gas mixture (N$_2$:CO$_2$, 95:5, previously passed through a hot, reduced copper furnace to remove all traces of O$_2$) and sealing each tube under an atmosphere of anoxic gas. Tubes (containing about an equal volume of medium and gas headspace) are stoppered and then autoclaved in a tube press. Sterile tubes can be stored outside the anoxic glove box and inoculated using standard "Hungate-type" techniques.

Identification

The major identifying feature of species of Heliobacteriaceae is the presence of Bchl *g* (Fig. 1). Organisms containing Bchl *g* show in vivo absorption maxima at 788–790 nm (Fig. 2; see also Table 1). However, one must be very cautious in interpreting spectra from heliobacterial enrichment cultures because of the ease with which Bchl *g* is converted to a form of chlorophyll *a* upon exposure to oxygen and light; this conversion greatly affects absorption spectra (Beer-Romero et al., 1988, see also Fig. 2). For example, the small peak at 670 nm, which is always present in spectra of heliobacteria and presumably due to the hydroxychlorophyll *a* in their reaction center (Amesz, 1995), can become the major peak following exposure of intact cells to light in air (Fig. 2). This is a problem, of course, because it could lead one to erroneously conclude that the enrichment contained cyanobacteria instead of heliobacteria. Thus, the best way to perform spectra on cells from enrichment cultures (or pure cultures for that matter) is to transfer cells directly into a pre-reduced (0.05% ascorbate) viscous medium (60% w/v sucrose or 30% w/v bovine serum albumin) within an anoxic glove box. Following this, the mixture should be transferred to a cuvette within the glove box, mixed well, and then removed from the glove box, and the spectrum recorded immediately. As previously mentioned, definitive determination of Bchl *g* is proof that one has enriched species of Heliobacteriaceae.

On plates, colonies of heliobacteria are brown green in color and show either a flattened or slightly raised center with entire or spreading margins, depending on the strain (Stevenson et al., 1997; Fig. 10). However, because of the Bchl *g* → chlorophyll *a* transition mentioned above, colonies of heliobacteria will, like liquid cultures, change color from brown green to emerald green within an hour or so of exposure to air; during this time, cell viability decreases significantly.

Cells of species of heliobacteria are fairly large rods, 1 μm × 6–10 μm (Figs. 5–9). Cells of the alkaliphile *Heliorestis daurensis* form short filaments 8–20 μm long (Bryantseva et al., 1999; Fig. 11). Most isolates of heliobacteria are motile by flagellar means, either polar, subpolar or peritrichous (Fig. 8). In young pure cultures or in enrichments, slowly motile cells can be observed by phase microscopy. These tend to stop moving within a half-hour or so, probably because of oxygen toxicity. Cultures of many heliobacteria form spheroplasts in early stationary phase and so nonmotile rounded structures are commonly observed microscopically. Further features for identifying species of heliobacteria include the following: Gram-stain (negative); electron
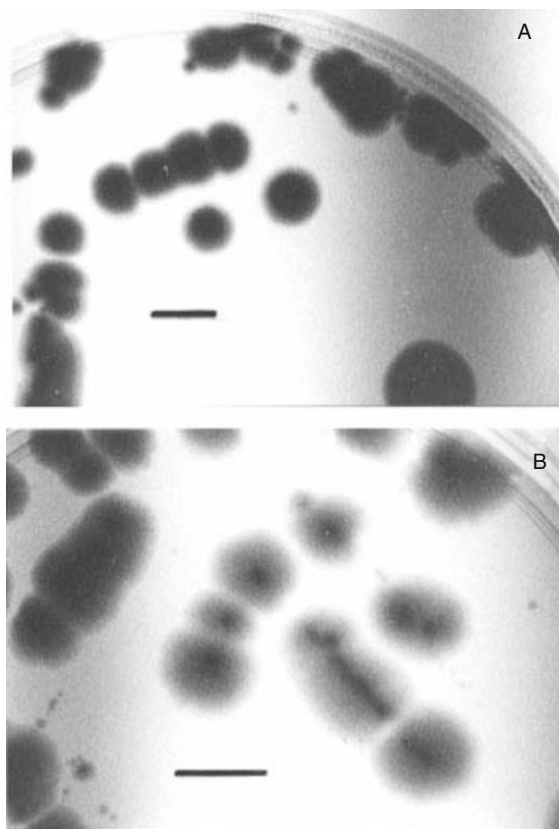
Fig. 10. Colonies of heliobacteria. A) Colonies of strain THAI 15P1, which produces nonspreading colonies with entire margins. B) Colonies of strain THAI 13P1, which produces spreading colonies with irregular margins. Cells of both strains were grown phototrophically on plates of medium PYE. Marker bars, 3 μm. (From Stevenson et al., 1997, with permission.)
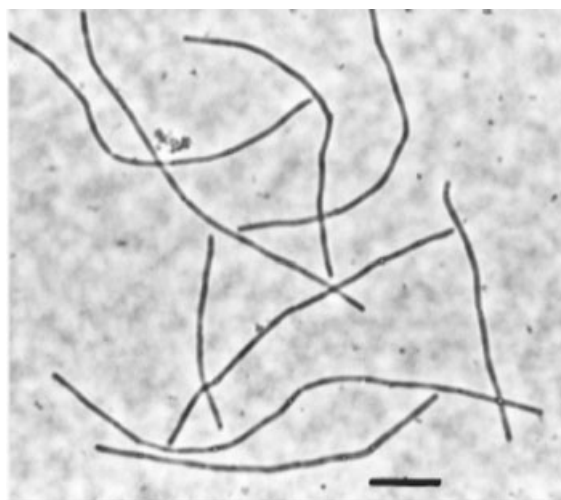
Fig. 11. Phase-contrast photomicrograph of cells of *Heliorestis daurensis* strain BT-H1. Cells are long rods to short filaments that display a slow swimming motility. Marker bar, 10 μm.

microscopy (thin, often "studded" cell wall and absence of internal photosynthetic membranes, Fig. 9); and lack of aerobic growth. However, it should be reemphasized that Bchl μ*g* is the definitive, and only reliable, indicator of heliobacteria.

Endospores can apparently be produced by all heliobacteria. This conclusion rests on the fact that cloned endospore-specific genes, such as the *ssp*1 gene from *Bacillus* or *Clostridium* species (Setlow, 1988), hybridize to DNA from all heliobacteria tested (L. K. Kimble and M. T. Madigan, manuscript in preparation). Although large sporulating rods, presumably heliobacteria, are a common observation in enrichment cultures (Stevenson et al., 1997), formation of endospores in pure cultures of heliobacteria occurs only sporadically, if at all. Thus, whereas the presence or absence of sporulating cells in an enrichment should not be taken as evidence for or against successful enrichment of heliobacteria, enrichments that contain Bchl μ*g* definitely contain heliobacteria.

Preservation

For short-term (weeks to months) storage of heliobacteria, "stabs" of medium PYE can be established and incubated in the light in anoxic jars. Once fully grown, stab cultures can be stored for several months in anoxic jars or in an anoxic chamber exposed to low light. For unknown reasons, stab cultures of heliobacteria retain viability much longer than do liquid cultures stored under the same conditions.

Freezing or lyophilizing cell suspensions of heliobacteria is more problematic, but has been done successfully. For freezing, cultures grown to mid-exponential phase on medium PYE should be mixed with an equal volume of pre-reduced medium PYE containing 10% (v/v) DMSO in sterile snap-cap disposable plastic tubes, and then kept at 0°C for 10 min. Tubes should then be stored at −80°C (or lower) in a freezer or at −196°C in liquid nitrogen. Lyophilization of dense suspensions of heliobacteria by the double vial method (Gherna, 1981) apparently also has been successful (R. L. Gherna, personal communication) but the author has no direct experience with this.

Recovery of frozen cultures is by no means a sure thing. In this laboratory, successful recovery has averaged only about 50%, and thus when a strain is set up to be frozen away, several replicate vials should be prepared. To recover cells from the frozen state the entire contents of the quickly thawed suspension should be transferred immediately to an anoxic tube of medium PYE, sealed under N_2:CO_2, and the tube kept in darkness for several hours before placing it in the light. If viable cells are present, growth should commence within one week. Also, for unknown reasons, certain species of heliobacteria freeze better than others. For example, although recovery of frozen cultures of *Heliorestis daurensis* is close to 100%, recovery of frozen cells of *Heliobacillus mobilis* is much less than 50% (M. T. Madigan, unpublished observations).

Physiology

As far as is currently known, heliobacteria are obligate anaerobes. However, they can grow both photo- and chemotrophically. Phototrophic growth occurs under anoxic conditions in the light and requires organic compounds as carbon sources, and thus growth is referred to as photoheterotrophic. However, unlike purple nonsulfur bacteria, also photoheterotrophs (Madigan, 1988), heliobacteria described to date catabolize only a short list of organic substrates including pyruvate, lactate, acetate, butyrate (+CO_2), ethanol (+CO_2), and yeast extract; *Heliobacterium gestii* also photometabolizes a few sugars (Table 1).

Pyruvate is the only compound universally used by heliobacteria (Table 1). Excellent photoheterotrophic growth of all known heliobacteria can be achieved in defined media containing pyruvate and biotin as a growth factor. With a few species, a reduced sulfur source for biosynthetic purposes is also needed (Table 1). Supplementing the medium with a small amount of thiosulfate, cysteine, methionine, thioglycollate, sulfide or yeast extract can satisfy the latter.

Photoautotrophic growth has not been demonstrated with any species of heliobacteria. If these organisms truly lack an autotrophic capacity, it would be a unique situation among phototrophic organisms. Curiously, however, many heliobacteria are quite sulfide tolerant, and when significant (1–2 mM) levels of sulfide are present in photoheterotrophic media, it is oxidized to elemental sulfur during growth (the sulfur appears in the medium as it does with green sulfur bacteria). However, in the presence of sulfide and with CO–/bicarbonate as sole carbon sources, photoautotrophic growth does not occur. Molecular hydrogen (H–) also fails to support autotrophic growth.

Concerning the capacity of heliobacteria for photoautotrophy, it is significant that no hybridization was observed in Southern blots of DNA from two species of heliobacteria with a ribulose bisphosphate carboxylase (RuBisCO) probe (F. R. Tabita, personal communication). RuBisCO is the key enzyme of the Calvin cycle, a widely distributed pathway of autotrophy in phototrophic and chemolithotrophic prokary-

otes (Tabita, 1995). Thus it is possible that the lack of photoautotrophy in the heliobacteria is due to the absence of an autotrophic CO_2 fixation pathway. On the other hand, maybe an alternative autotrophic pathway exists in the heliobacteria, but the necessary conditions for expression of the genes that encode it have not been found. More work on this problem is clearly needed.

Pyruvate-dependent chemotrophic (dark) growth of heliobacteria occurs with most species (Kimble et al., 1994). Chemotrophic growth requires anoxic conditions and is optimal in heavily buffered media containing high levels of pyruvate. Medium DPYE, designed for chemotrophic growth of heliobacteria, is medium PYE (see Isolation) modified to contain a final concentration of 60 mM pyruvate, 0.4% (w/v) yeast extract, 10 mM K_2HPO_4, and 40 mM 3-(N-morpholino) propane sulfonic acid (MOPS, Sigma, St. Louis) as buffer (final pH 7.3; Kimble et al., 1994). Chemotrophically grown cells of heliobacteria are fully pigmented; thus it can be concluded that, as for purple bacteria (Bauer, 1995), anoxia is the signal for derepression of pigment synthesis in heliobacteria.

Energy conservation during chemotrophic growth of heliobacteria occurs by pyruvate fermentation. None of the other carbon sources that support photoheterotrophic growth of heliobacteria (Table 1) support chemotrophic growth. Chemotrophically grown cultures acidify the medium and analyses have shown that acetate accumulates as a product during growth on pyruvate (Pickett et al., 1994; Kimble et al., 1994). It is thus likely that chemotrophic growth of heliobacteria occurs by fermentation of pyruvate through the activity of either pyruvate:ferredoxin oxidoreductase or pyruvate:formate lyase (or both); these enzymes cleave pyruvate to generate acetyl-CoA, which can be converted to acetate with formation of ATP (Thauer et al., 1977).

Ecology

The finding that heliobacteria reside mainly in soil points to a unique ecological role for these organisms in nature. Anoxygenic purple and green bacteria are primarily aquatic organisms (Pfennig, 1989), and although they occasionally can be enriched from soils, their ecological significance outside of aquatic environments is likely minimal (Pfennig, 1989). By contrast, heliobacteria clearly reside in soil (Stevenson et al., 1997) and employ at least one excellent strategy for survival there; unlike purple and green bacteria, heliobacteria can sporulate (see Phylogeny and Taxonomy) and this capacity proba-

bly allows them to withstand periods of unfavorable growth conditions in soil.

The apparent lack of autotrophic capacities in the heliobacteria (see Physiology) means that they are likely to be most active and abundant in the rhizosphere, where organic compounds excreted from plant roots would support their photoheterotrophic lifestyle. In this connection, the discovery that heliobacteria are widespread in paddy fields (Stevenson et al., 1997) is not surprising. Indeed, perhaps a truly symbiotic relationship exists between heliobacteria and rice plants-in exchange for fixed nitrogen, heliobacteria would obtain from the rice plants the carbon sources they need for biosynthesis and N_2 fixation. Nitrogen fixation by phototrophic organisms is known to be a major process in paddy soils (Buresh et al., 1980; Habte and Alexander, 1980) and heliobacteria are strong nitrogen-fixers (Kimble and Madigan, 1992a; Kimble and Madigan, 1992b; Madigan, 1995a). Thus, some (if not much) of the N2 fixation that occurs in paddy soils may be due to heliobacteria.

Study of the possible symbiosis between plants and heliobacteria would be a good area for future experimentation using molecular techniques like polymerase chain reaction (PCR) and fluorescent-in situ-hybridization (FISH). In this connection, collaborative work from my laboratory has yielded highly specific nucleic acid probes for the heliobacteria based on signature sequences in their 16S rRNA (L. A. Achenbach, J. R. Carey and M. T. Madigan, unpublished observation); such probes could be used in both of these techniques.

As regards N_2-fixation, it also should be mentioned that some heliobacteria, in particular the species *H. gestii*, contain alternative (non-molybdenum; non-Mo) nitrogenases. The alternative system in *H. gestii* is an iron (Fe)-only (type III) nitrogenase (Kimble and Madigan, 1992b; Loveless and Bishop, 1999); such an enzyme might be very useful to heliobacteria growing in paddy fields where Fe is typically abundant but where other heavy metals, such as Mo and vanadium (V), have leached out.

An enigma surrounding the ecology of heliobacteria is why phototrophic organisms, especially obligately anaerobic phototrophs that grow best at high light intensities, would inhabit soil in the first place. One might easily predict that soils in general would greatly restrict light penetration and that in other than waterlogged soils, anoxic conditions would be localized and transitory. However, soils are known to be quite heterogeneous in their chemical and physical properties and so the combination of light, anoxic conditions, and organic compounds necessary for photoheterotrophic growth of heliobacteria may be more common than we think

(Madigan manuscript submitted to Appl. Environ. Microbiol.). Again, this is an area where molecular probing might yield some interesting results.

The ecology of heliobacteria in microbial mats is another interesting question. Using molecular probes, it should be possible to track heliobacteria through mat cores to see where these organisms are most abundant. Gradients of light and O_2 can be quite steep in microbial mats (Ward et al., 1989) and thus heliobacteria are likely to be present only in certain zones. Heliobacteria may be ecologically significant in microbial mats in terms of N_2 fixation. For example, the thermophile *H. modesticaldum* can fix N_2 up to its maximum growth temperature of 57°C (Kimble et al., 1995) and thus may contribute to the nitrogen economy of a wide variety of hot spring microbial mats.

Applications

The major application of heliobacteria thus far has been as experimental tools for biophysical and biochemical studies of photosynthesis (Amesz, 1995). The reaction center photocomplex of heliobacteria is an excellent model for green plant photosystem I, and because of this, heliobacteria have been intensely studied in terms of the details of photosynthetic electron flow (Amesz, 1995; Schubert et al., 1998). Unfortunately, no genetic transfer system exists for any species of heliobacteria. But when such is developed, the ability of heliobacteria to grow chemotrophically in darkness should allow photosynthetically incompetent mutants to be isolated and studied, as has been done extensively with purple bacteria (Bauer, 1995). This, combined with a genetic system would allow for the first genetic probing of green plant photosystem I events in a bacterial system. However, despite the absence of a genetic transfer system, much progress has already been made using cloned DNA from heliobacteria to complement mutants in other photosynthetic microorganisms (Bauer, 1995; Xiong et al, 2000). New and exciting information on the unity and diversity of photosynthesis has emerged from such studies (Xiong et al., 2000).

Future agricultural applications of heliobacteria could emerge from a better understanding of their nitrogen-fixing activities in soils. As previously mentioned (see Ecology), heliobacteria are primarily soil organisms and may thus be ecologically significant contributors of fixed nitrogen to soil ecosystems, paddy soils in particular. If this is true, then heliobacteria would be agents of soil fertility for the major food staple of over one-half the world's population. However, under any

conditions, the fact that the ecology of heliobacteria is closely linked to soil and the fact that all major representatives fix molecular nitrogen (Kimble and Madigan, 1992a; Kimble and Madigan, 1992b; Madigan, 1995a), means that heliobacteria likely possess features of interest to plant biologists and speaks well for the future agricultural significance of the group.

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CHAPTER 1.3.2

Pectinatus, *Megasphaera* and *Zymophilus*

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Introduction

First described in the late 1970s (Lee et al., 1978; Weiss et al., 1979), *Pectinatus* and *Megasphaera* are both anaerobic, Gram-negative contaminants of unpasteurized beer. The anaerobic, Gram-negative *Zymophilus*, isolated from pitching yeast, was described in 1990 along with a new *Selenomonas* sp. (Schleifer et al., 1990). The new genus and species *Pectinatus cerevisiiphilus* (Lee et al., 1978) and new species *Megasphaera cerevisiae* (Engelmann and Weiss, 1985) were proposed on the basis of genetic and phenotypic differences existing between these new isolates and related genera or species described in *Bergey's Manual of Systematic Bacteriology* (Rogosa, 1984). The new species *Pectinatus frisingensis*, although already isolated in 1978 and identified as *P. cerevisiiphilus* in 1981 (Haikara et al., 1981b), was finally established in 1990 (Schleifer et al., 1990) simultaneously with the description of a new genus comprising the two species *Zymophilus raffinosivorans* and *Zymophilus paucivorans*. Analysis by 16S rRNA sequence comparisons, DNA/DNA hybridizations and phenotypic characterization has revealed the phylogenetic positions of *Pectinatus*, *Megasphaera* and *Zymophilus*. These genera belong to the *Sporomusa* subbranch of the Gram-positive Clostridium subphylum, indicating possible Gram-positive ancestors. The genetic relationship between the species of the Gram-negative, anaerobic brewery bacteria still needs to be refined.

Their anaerobic nature largely explains the simultaneous occurrence of *Pectinatus* and *Megasphaera* species in beer (in many countries) and the detection of *Zymophilus*. The dissolved oxygen content present during brewing, particularly of beer, has decreased considerably in recent years. Minimal access to oxygen is a brewing technology advance intended to improve the important chemical stability of beer. As a consequence, these strict anaerobes are mainly encountered as contaminants in large, modern breweries equipped with effective filling tech-

nology (Chelack and Ingledew, 1987; Seidel et al., 1979). Another reason might be the growing tendency in industry to produce more "natural" beer without pasteurization. On the other hand, the most effective cleaning and disinfection practices in use have managed to eliminate more rapidly growing contaminants, thus providing growth opportunities for anaerobic beer spoilers.

Phylogeny

Pectinatus

The phylogenetic position of *Pectinatus* has been confirmed during recent years (Schleifer et al., 1990; Motoyama and Ogata, 2000b; Ludwig et al., 1992; Both et al., 1992; Willems and Collins, 1995; Sawada et al., 1999; Klugbauer et al., 1992). The 16S rRNA, 23S rRNA and the ATPase β-subunit gene sequences, the 16S-23S rDNA intergenic spacer regions as well as DNA-DNA hybridization have been used as phylogenetic markers. *Pectinatus* species, along with *Selenomonas*, *Megasphaera*, *Sporomusa*, *Veillonella* and *Zymophilus*, belong to the *Sporomusa* subbranch of the *Clostridium subphylum* of Gram-positive bacteria (Schleifer et al., 1990; Willems and Collins, 1995). Moreover, *Pectinatus* species have been classified as relatives of Gram-negative, anaerobic bacteria such as *Centipeda periodontii* (Sawada et al., 1999), *Dialister pneumosintes* (Willems and Collins, 1995), *Schwarzia succinivorans* (Van Gylswyk et al., 1997), *Acidaminococcus fermentans* (Both et al., 1992) and some *Anaerovibrio* species (Strömpl et al., 1999). According to the 16S rRNA sequences, the similarity between *P. frisingensis* and *P. cerevisiiphilus* was 95%, the nearest other species (similarities with *Pectinatus* spp.) being three *Selenomonas* species (88–89%), *Clostridium quercicolum* (87–88%), *Sporomusa paucivorans* (86–88%), *Veillonella parvula* (86–87%) and *Megasphaera elsdenii* (86%; Willems and Collins, 1995). The phylogenetic studies of Motoyama and Ogata (2000b)

showed that the 16S–23S rDNA intergenic spacer regions of two *Pectinatus* spp., two *Zymophilus* spp. and one *Selenomonas* sp. were of two molecular sizes (long and short). The DNA sequence homologies of the long spacer region indicated that *Pectinatus* spp. were more closely related to *Selenomonas lacticifex* than to *Zymophilus* spp. The dendrogram of the short spacer region, however, did not agree with that of the long spacer region, which according to the authors better reflects the phylogenetic position. Moreover, analysis of the short spacer region did not correspond to that of the 16S rRNA sequence published by Schleifer et al. (1990), who found *S. lacticifex* was more closely related to *Zymophilus* spp. than to *Pectinatus* spp. Notably, the order of the alanine tRNA/isoleucine tRNA genes present in the long spacer regions of *Pectinatus* and *Selenomonas* strains was the reverse of what was previously reported (Gürtler and Stanisich, 1996).

From 16S rRNA analysis, it is quite clear that the genus *Pectinatus* does not belong to the family Bacteroidaceae, as originally suggested (Lee et al., 1978), because the genus *Bacteroides* and related genera represent a distinct phylum within the phylogenetic tree of bacteria (Schleifer et al., 1990; Willems and Collins, 1995). The unrelatedness is also evident on the basis of the lipopolysaccharide composition of representatives of the genus *Pectinatus* (Helander et al., 1983; Helander et al., 1992).

Megasphaera

Megasphaera, like *Pectinatus*, *Zymophilus*, *Sporomusa* and *Selenomonas* species, belongs to the group of bacterial species with Gram-negative cell walls within the phylum of Gram-positive bacteria (Stackebrandt et al., 1985; Schleifer et al., 1990).

Doyle et al. (1995) have clarified the phylogenetic position of *Megasphaera cerevisiae* by determining the nucleotide sequence of the small subunit 16S rRNA of the bacterium. Analysis confirmed the phylogenetic position of *M. cerevisiae* as a sister taxon of *M. elsdenii* within the obligately anaerobic, Gram-negative cocci. The phylogenetic tree (obtained by applying the neighbor-joining method and parsimony analyses) demonstrated the relationships between *M. cerevisiae* and organisms of the *Sporomusa* group (Doyle et al., 1995).

Both et al. (1992) studied the phylogenetic position of *Acidaminococcus fermentans* in the *Sporomusa* cluster containing Gram-negative bacteria, i.e., *Sporomusa*, *Megasphaera*, *Selenomonas*, *Butyrivibrio*, *Pectinatus* and *Zymophilus*. Within this cluster, the relationship between species of *Acidaminococcus* and *Megasphaera*, described to be members of *Veillonellaceae*, was obvious but did not exclude other Gram-negative organisms. The phylogenetic analysis of *Dialister pneumosintes* (formerly *Bacteroides pneumosintes*) revealed that the closest relatives of this species within the *Sporomusa* subbranch of *Clostridium subphylum* were *Megasphaera elsdenii* and *Veillonella species* (Willems and Collins, 1995).

Zymophilus

According to the 16S rRNA nucleotide sequences, *Zymophilus* spp. belong to the *Sporomusa* subdivision of the *Clostridium subphylum* (Schleifer et al., 1990; Willems and Collins, 1995; Both et al., 1992). The similarity between two *Pectinatus* species and *Z. paucivorans* was 88%, which was also their similarity to *Selenomonas lacticifex* (Willems and Collins, 1995). On the basis of the DNA sequence homologies of long 16S-23S intergenic spacer regions, *S. lacticifex* was more closely related to *Pectinatus* spp. (51%) than to *Zymophilus* spp. (40%; Motoyama and Ogata, 2000b).

Taxonomy

Taxonomic Differentiation of *Pectinatus*

Pectinatus cerevisiiphilus was originally suggested to belong to the family Bacteroidaceae consisting of anaerobic, nonsporeforming Gram-negative rods (Lee et al., 1978) and later described in {*Bergey's Manual of Systematic Bacteriology*} (Lee, 1984). During the 1980s several genera (e.g., *Pectinatus* and *Selenomonas*) in this family underwent taxonomic revisions and, owing to their closer affinity (Schleifer et al., 1990; Willems and Collins, 1995), were transferred to the *Clostridium subphylum* of the Gram-positive bacteria.

Simultaneous with the description of the new *P. cerevisiiphilus* ATCC 29359T, another strain (VTT E-79100; later P. frisingensis ATCC 33332T) was isolated in Finland and identified as the same species on the basis of its physiological and biochemical characteristics (Haikara et al., 1981b). However, this strain as well as many other new isolates (when compared to *P. cerevisiiphilus*) showed clear-cut differences in serological characteristics (see Identification), cell-surface-protein patterns and immunoblot analysis (Haikara, 1983; Hakalehto et al., 1984; Hakalehto and Finne, 1990). DNA/DNA hybridization studies also revealed two distinct groups among *Pectinatus* strains isolated from beer (Haikara, 1989; Schleifer et al., 1990). Schleifer et al. (1990), who also described the new species

P. frisingensis, determined the similarity value of 16% between strains ATCC 29359 and ATCC 33332. Only small differences existed between the G+C content of the DNA of *Pectinatus* species/strains (38 to 41 mol%; see Physiology; Back et al., 1979; Haikara et al., 1981b; Kirchner et al., 1980; Schleifer et al., 1990).

In addition to a low degree of genotypic similarity between the two species, they could be phenotypically distinguished (Schleifer et al., 1990). Many characteristic differences were observed in the utilization of carbon sources, e.g., *P. cerevisiiphilus* degraded xylose and melibiose, but not cellobiose and *N*-acetyl-glucosamine, whereas in the case of *P. frisingensis* the situation was the reverse (see Physiology; Haikara, 1991; Schleifer et al., 1990). Moreover, Schleifer et al. (1990) amended the description of *P. cerevisiiphilus* because the phenotypic properties of *P. cerevisiiphilus* ATCC 29359 T were not in agreement with the original description (Lee et al., 1978).

Cell Envelope Components

FATTY ACIDS The cellular fatty acid composition is similar in type strains of *P. cerevisiiphilus* and *P. frisingensis*. The fatty acids are heavily dominated by odd-numbered fatty acids; 11:0, 15:0, 17:1, 18:cyc and 13:0(3-OH) were the main fatty acids detected in both species (Helander and Haikara, 1995). Alk-1-enyl chains with chain lengths of 15 to 18 also were found, indicating the presence of plasmalogens common in strict anaerobic bacteria. In addition to the major hydroxy acid component (*R*)-13:0(3-OH), both *Pectinatus* species were shown to contain, in minor amounts, five 3-hydroxy acids with chain lengths of 11 to 15 carbons. These included the unsaturated acid 13:1(3-OH), the occurrence of which is limited to *Pectinatus*. As discussed by Helander and Haikara (1995), and Strömpl et al. (1999), because fatty acid 13:0(3-OH) is not in the MIDI database utilized for fatty acid-based identification of anaerobic bacteria, this fatty acid has been erroneously identified as 14:0 dimethylacetal (DMA) in several bacterial genera, including *Pectinatus* (Moore et al., 1994).

PEPTIDOGLYCAN *Pectinatus* possesses cross-linked meso-diaminopimelic acid-containing peptidoglycan, with covalently linked diamine (cadaverine or, rarely, putrescine) in the peptide subunit of the peptidoglycan (Schleifer et al., 1990). With the aid of monoclonal antibodies isolated on the basis of their binding to *P. cerevisiiphilus* peptidoglycan, Ziola et al. (1999) found a peptidoglycan structure that they suggested was common to several species of anaerobic beer spoilage bacteria, including *P. frisingensis*, *Sele-*

nomonas lacticifix, *Zymophilus paucivorans* and *Z. raffinosivorans*.

LIPOPOLYSACCHARIDES The cell surface lipopolysaccharides (LPS) of the type strains *P. cerevisiiphilus* ATCC 29359 and *P. frisingensis* ATCC 33332 have been characterized in detail. Several exceptional properties are assigned to *Pectinatus* LPSs, including the production of at least two distinct types of LPS by one strain (Helander et al., 1992), the presence of the phosphorylated disaccharide α-D-GlcpN-(1'-4)-Kdo (3-deoxy-D-manno-oct-2-ulopyranosonic acid) in the LPS core (Helander et al., 1993), the resistance of the lipid A-polysaccharide linkage to acid (Helander et al., 1994), and the predominance of furanosidic 6-deoxyhexoses in the O-specific chains (Senchenkova et al., 1995). The lipid A backbone of *P. cerevisiiphilus* and *P. frisingensis* is composed of the common bisphosphorylated β1'-6-linked glucosamine (GlcN) disaccharide, with almost quantitative substitution of the ester-linked phosphate by 4-amino-4-deoxyarabinose (Helander et al., 1994). It was later shown that the glycosidically linked phosphate of *Pectinatus* also carries minor amounts of this aminopentose, analogous to the structure found in *Klebsiella pneumoniae* O3 lipid A (Helander et al., 1996). The fatty acids in *Pectinatus* lipid A comprises two amide-linked and two ester-linked 13:0(3-OH). The hydroksyl groups of the fatty acids linked to the non-reducing GlcN, carry the fatty acid 11:0 or, to a small extent, 13:0 in acyloxyacyl linkage. Whereas 13:0(3-OH) is the main hydroxy acid in *Pectinatus*, the minor 3-hydroxy acids are also most probably constituents of the lipid A, although their position has not been determined. Complete structures of the core oligosaccharides in *Pectinatus* LPSs are not known, but each strain presumably elaborates two structurally distinct cores, only one of which carries polymeric O-specific chains; these two LPS populations can be separated from each other during processing of initial phenol/chloroform/petroleum ether extracts (Helander et al., 1992). The O-specific chain of *P. cerevisiiphilus* is composed of repeating disaccharides of 1 → 2 linked β-D-fucofuranose and α-D-glucopyranose, whereas the repeating unit of *P. frisingensis* O-specific chain is a branched tetrasaccharide consisting of a single sugar type, the rare 6-deoxy-L-altrofuranose (Senchenkova et al., 1995). It is worthy of note that the O-specific chains of *Pectinatus* are highly labile towards acid, whereas the normally acid-labile lipid A-core linkage is exceptionally stable. The reason for the latter property is unknown at present, but the presence of acid-stable linking sugars such a 2-octulosonic acid has been excluded in *Pectinatus* LPSs (Helander et al.,

1994). The LPSs of both species of *Pectinatus* exhibit the biological potency of classical endotoxins (Helander et al., 1984).

RIBOTYPING The ribotyping fingerprinting technique has proved to be a useful tool for characterization and identification of beer spoilage bacteria (Storgårds et al., 1998; Satokari et al., 2000; Motoyama et al., 1998; Motoyama et al., 2000c; Suihko and Haikara, 2001). Motoyama et al. (1998) used three different restriction enzymes (i.e., EcoRI, HindIII and BamHI) for ribotyping of *Pectinatus cerevisiiphilus* and *P. frisingensis* strains. When a combination of all these enzymes was used, all 34 *P. frisingensis* strains fell into 1 of 17 ribotypes. Five *P. cerevisiiphilus* strains were classed as one of three types with single enzymes, the composite ribotypes not showing greater definition. Suihko and Haikara (2001) used EcoRI enzyme for characterization of 24 *P. frisingensis* and 8 *P. cerevisiiphilus* strains and used 16S rDNA sequencing of selected strains for confirming the ribotyping. *Pectinatus frisingensis* strains were divided into nine different ribotypes and *P. cerevisiiphilus* strains into five, allowing identification below the species level. Interestingly, these techniques also revealed a possible new species within the genus *Pectinatus*.

Taxonomic Differentiation of *Megasphaera*

Anaerobic Gram-negative cocci were assigned in *Bergey's Manual of Determinative Bacteriology* to the family Veillonellaceae (Rogosa, 1974). On the basis of differential characteristics of the genera *Veillonella*, *Acidaminococcus* and *Megasphaera* of this family, the beer isolates could be assigned to the genus *Megasphaera* (Weiss et al., 1979). The abilities to ferment carbohydrates and to produce volatile fatty acids containing 5–6 carbon atoms are the most distinctive characteristics of the genus *Megasphaera*. The only previously known species in this genus was a rumen microbe, *M. elsdenii* (Rogosa, 1971; Rogosa, 1984). The G+C content of the DNA of this species is 53.6 mol%. The corresponding G+C values of 12 brewery isolates ranged from 42.4 to 44.8 mol% (Table 1; Engelmann and Weiss, 1985), indicating that there is no genomic relationship between the *Megasphaera* species isolated from beer and *M. elsdenii*. DNA/DNA hybridization studies also supported this finding and showed that all isolates from beer belong to a single genospecies (Engelmann and Weiss, 1985), the similarity within this group being over 72%. Moreover, no genomic relatedness was detectable between *Veillonella parvula* and the beer isolates, and hence Engelmann and Weiss (1985) proposed a new species named *M. cerevisiae*.

The 16S ribosomal RNA gene analysis results have later confirmed the phylogenetic position of *M. cerevisiae* as a sister taxon of *M. elsdenii* within the obligately anaerobic, Gram-negative cocci and facilitated the development of specific polymerase chain reaction (PCR) primers for early detection of this spoilage organism (Doyle et al., 1995; Satokari et al., 1998; Juvonen et al., 1999; Sakamoto et al., 1997).

Composition of the Cell Envelope

FATTY ACIDS The cellular fatty acids of *M. cerevisiae* and *M. elsdenii* have been investigated, and shown to be almost identical (Johnston and Goldfine, 1982; Helander and Haikara, 1995). The main fatty acid components are 12:0, 16:0, 16:1, 18:1, 17:cyc, 19:cyc, 12:0(3-OH) and 14:0(3-OH), and alk-1-enyl chains instead of acyl chains are detected to a considerable extent (14% of

Table 1. Characteristics of Gram-negative, anaerobic genera isolated during the brewing processes.

Characteristics	Genus			
	Pectinatus	*Zymophilus*	*Selenomonas*	*Megasphaera*
Curved rods	+	+	+	−
Cocci	−	−	−	+
Products of glucose or fructose fermentation				
Acetate	+	+	+	(+)
Acetoin	+	−	ND	−
Butyrate	−	−	−	+
Lactate	−	−	− or +[a]	−
Propionate	+	+	+	(+)
Succinate	−	−	−	−
Valerate and caproate	−	−	−	+
G+C content (mol%)	38–41	38–41	48–58	42–45

Symbols: +, positive reaction; −, negative reaction; (+), minor products; ND, not determined.
[a]*S. Lacticifex* +.
Adapted from Schleifer et al. (1990).

total fatty acids); alk-1-enyl chains are indicative of plasmalogen lipids, as demonstrated previously by Kaufman et al. (Kaufman et al., 1988; Kaufman et al., 1990) for *M. elsdenii*. No information is available concerning the LPS composition of the genus *Megasphaera*.

PEPTIDOGLYCAN The cell surface protein pattern and the chemical composition of *M. cerevisiae* LPS is not known. However, Engelmann and Weiss (1985) analyzed the amino acid composition of the cell wall peptidoglycans of *Megasphaera* spp. The peptidoglycan of *M. cerevisiae* as well as that of *M. elsdenii* was of the meso-diaminopimelic acid direct type and contained putrescine residues.

RIBOTYPING Automated ribotyping has been used for characterization of 13 *Megasphaera cerevisiae* strains using three restriction enzymes *Eco*RI, *Pst*I and *Pvu*II (Suihko and Haikara, 2001). Using a combination of the enzymes, the strains could be divided into seven ribotypes, of which one probably represented a novel species within the genus.

Taxonomic Differentation of *Zymophilus*

The Gram-negative, anaerobic bacteria isolated from pitching yeast were preliminarily classified as *Pectinatus* (Back, 1981; Haikara, 1989). The taxonomic rearrangement of anaerobic, Gram-negative, rod-shaped bacteria from breweries revealed a new genus comprising the two species *Zymophilus raffinosivorans* and *Z. paucivorans* and a new species *Selenomonas lacticifex* (Schleifer et al., 1990). The G+C contents of DNAs of *Zymophilus* and *Pectinatus* spp. were similar (i.e., 38–41 mol%), different from those of *Selenomonas* and *Megasphaera* spp. (Table 1). The DNA/DNA hybridization studies showed that *Zymophilus* species formed a distinct genospecies compared to *Pectinatus* and *Selenomonas* spp. *Zymophilus paucivorans* strains exhibited high similarity values with each other (>75%), and also a distinct relationship (44–48%) to *Z. raffinosivorans* strains (Schleifer et al., 1990).

CELL WALL The cell wall of *Zymophilus* spp. as well as that of *Pectinatus* and *Selenomonas* species contained the directly cross-linked meso-diaminopimelic acid peptidoglycan type (Schleifer et al., 1990). In addition, the α-carboxyl group of D-glutamic acid was substituted predominantly by cadaverine, and in some strains a minor amount of cadaverine was replaced by putrescine. Such a peptidoglycan type containing a diamine-substituted glutamic acid has previously been found in strains of *M. cerevisiae* (Engelmann and Weiss, 1985), *Veillonella* species

and *Centipeda periodontii*, indicating the relationship of *Zymophilus* spp. to the subdivision of species with Gram-negative cell walls within the phylum of Gram-positive eubacteria. Lipid F was also found as a characteristic cellular compound (Schleifer et al., 1990).

RIBOTYPING *Zymophilus raffinosivorans* and *Z. paucivorans* have been characterized by digestion with the restriction enzyme *Eco*RI (Motoyama et al., 1998; Suihko and Haikara, 2001). These species were completely different from those of the genus *Pectinatus*, indicating that the patterns of fragments were species-specific.

Table 2 summarizes the characteristics of Gram-negative, anaerobic genera isolated from brewing processes. The physiological differentiation can be based on the acetoin production of *Pectinatus*, lactic acid production of *Selenomonas lacticifex* or on the coccoid cell morphology of *Megasphaera*. For differentation on the species level, the utilization of carbohydrates must be determined (Table 2).

Habitat

Pectinatus and *Megasphaera* constitute an important group of spoilage bacteria of unpasteurized packaged beer. *Pectinatus* contaminations have occurred in most European countries and in the United States. *Megasphaera cerevisiae* is apparently less widespread and has been reported only in Germany, Australia and Finland. The natural environment of anaerobic beer spoilage organisms and the source of contaminations are not known. Most of the strains described and characterized (to date) have been isolated almost exclusively from spoiled beer. The occurrence of *Pectinatus* and *Megasphaera* in pitching yeast has been reported (Back, 1981; Seidel, 1985; Haikara, 1989). However, some of these isolates have later been reclassified as a new *Selenomonas lacticifex* species (Schleifer et al., 1990). *Pectinatus* has also sporadically been found in lubrication oil mixed with beer, in drainage and water pipe systems, in the air of the filling hall, in the filling machine, on the floor of the filling hall, in condensed water on the ceiling, in chain lubricants, and in steeping water of malt before milling (Back et al., 1988; Dürr, 1983; Haukeli, 1980; Lee et al., 1980; Soberka et al., 1986). The *Pectinatus* findings in Finland (Haikara, 1991) also have concentrated in the filling hall, indicating water is a most likely source of contamination. Moreover, it is obvious that *Pectinatus*, despite its anaerobic nature, can survive in aerosols and be transferred via the air into beer. The detection of *Pectinatus* in air near unclear bottles

Table 2. Differential characteristics of anaerobic Gram-negative bacteria isolated from spoiled beer and from pitching yeast.

	Species				
	Pectinatus cerevisiiphilus	*Pectinatus frisingensis*	*Zymophilus raffinosivorans*	*Zymophilus paucivorans*	*Megasphaera cerevisiae*
Acid produced from:					
Ribose	+	+	+	+	−
Xylose	+	−	+	−	−
Rhamnose	+	+	+	−	−
Mannitol	+	+	+	+	−
Sorbitol	+	+	+	+	−
Xylitol	−	d	+	−	ND
Lactose	−	−	+	−	−
Maltose	−	−	+	+	−
Sucrose	−	−	+	+	−
Cellobiose	−	+	+	+	−
Inositol	−	+	+	−	ND
Raffinose	−	−	+	−	−
Melibiose	+	−	+	−	ND
N-acetyl-glucosamine	−	+	+	−	ND
Acetoin production	+	+	−	−	−
G+C content (mol%)	38–41	38–41	38–41	39–41	42–45

Symbols: +, 75% or more of the strains are positive; −, 75% or more of the strains are negative; d, 26 to 74% of the strains are positive; ND, not determined.
Adapted from Schleifer, 1990; Haikara, 1991; Engelmann, 1985.

and close to the bottling machine in the filling hall indicates the possibility that air may be a contamination source (Dürr, 1983; A. Haikara, unpublished observations; Hakalehto, 2000). Hakalehto (2000) clearly demonstrated the general presence of *Pectinatus* in different parts of the brewery and considered his findings as a proof that the contaminating strains were in fact permanent inhabitants rather than occasional invaders of the brewery and that they find niches in the factory where they can survive.

Rather high frequencies of contaminations caused by *Pectinatus* and *Megasphaera* were reported in the 1980s (Haikara, 1991). Since then at least the reported cases have declined although the "anaerobic" filling technique and the low oxygen content of beer increasingly favor the growth of anaerobic beer-spoilage organisms. It has been somewhat controversial whether *Pectinatus* has resided in breweries over longer periods of time, or is a recent arrival with raw material. For example, on the basis of the occurrence D-fucose in the O-specific chains of *Pectinatus* lipopolysaccharides, it was speculated (Helander et al., 1992) that *Pectinatus* originated from plant material, inasmuch as 6-deoxy D-hexoses are uncommon in nature except in plant pathogenic Gram-negative bacteria. The O-specific sugar in *P. frisingensis*, however, later proved to be 6-deoxyaltrose in the L configuration (Senchenkova et al., 1995), showing that the presence of D-6-deoxyhexoses is not an obligatory component within the genus.

The strictly anaerobic *Zymophilus raffinosivorans* and *Z. paucivorans* are very rare

brewery contaminants and hitherto have been isolated only in Germany from pitching yeast and brewery waste (Seidel-Rüfer, 1990; Schleifer et al., 1990). *Zymophilus raffinosivorans* is considered a potential spoilage organism, because it can grow in beer if the pH is high (i.e., around 5; Seidel-Rüfer, 1990). *Zymophilus paucivorans* is regarded as harmless owing to its inability to grow below pH 6.

Growth of *Pectinatus* and *Megasphaera* in Beer

It has been observed in practice and in laboratory tests that beer with low alcohol content (<2.25% w/v) is more prone to *Pectinatus*, and especially to *Megasphaera*, contamination than beer with higher alcohol content (Haikara, 1984; Haikara and Lounatmaa, 1987). The rate of spoilage is inversely dependent on the alcohol content of beer. The growth of *Megasphaera* is already restricted in commercial beer with an alcohol content of 3.5% (w/v). *Pectinatus* bacteria are more alcohol-tolerant, growing rather well in beer with higher alcohol contents (3.7–4.4% w/v), although the growth is slower than in low-alcohol beer. As reported earlier, *Pectinatus* does not grow in strong beer with an alcohol content exceeding 5.2% (w/v; Haikara et al., 1981b; Haukeli, 1980; Kirchner et al., 1980; Seidel et al., 1979).

The low pH of beer is one of most important characteristics affecting the growth of microorganisms in beer. The higher the pH of beer, the

more susceptible it is to *Megasphaera* contamination. *Pectinatus* is very acid-tolerant. A pH value of 4.1 is required for retardation of its growth, whereas the normal beer pH of 4.5 already restricts the growth of *Megasphaera* (Haikara, 1984; Haikara and Lounatmaa, 1987). In German Alt beer (pH 4.0), the growth of *Megasphaera* is prevented, but *Pectinatus* can still grow slowly in it (Kirchner et al., 1980; Seidel et al., 1979).

Hop bitter substances generally restrict the growth of microorganisms in beer. However, both *Pectinatus* and *Megasphaera* are rather tolerant of hops, inasmuch as growth occurs in beer with bitterness in the range of 33–38 European Brewery Convention units (Back, 1981; Kirchner et al., 1980; Seidel et al., 1979). The growth of *Pectinatus* and *Megasphaera* bacteria in beer is dependent on the combined effects of several factors, the oxygen content of beer being the most decisive. The dissolved oxygen content as well as the volume of air in the headspace has decreased considerably in recent years due to advances in filling technology. At present an air volume of 1 ml or less in the headspace is easily achievable, as is an oxygen content of as little as 0.3 mg/liter (Seidel et al., 1979).

It has been reported that treatment at 58–60°C for one minute, which is less than normal pasteurization treatment, is sufficient to kill *Pectinatus* bacteria (Lee et al., 1981; Haukeli, 1980). Watier et al. (1995) studied the heat resistance of *Pectinatus* spp. in wort and culture medium, confirming the low D–60 values (a measurement of heat resistance at 60°C) for *Pectinatus* in wort. Some differences existed between species depending on the pH and the medium used.

The elimination of contaminations caused by anaerobic beer spoilers requires adequate cleaning, disinfection and sterilization of equipment susceptible to contamination. Lee et al. (1981) reported that iodine and chlorine were effective disinfectants for the control of *Pectinatus* spp. In our experiments, in which different disinfectants were tested, peracetic acid (0.75%, 1 min) and formaldehyde (0.3%, 1 min) were most efficient (Haikara, 1984). Disinfectants with iodine as the active compound proved to be ineffective. *Megasphaera* was killed most easily with oxidizing agents, iodophor having no effect (Haikara, 1991). Quaternary ammonium compound (0.2%) killed *Megasphaera* cells in one minute.

The detection of *Pectinatus* in the air of the handling area of dirty bottles has been proposed as one simple way to eliminate at least this source of contamination: treatment of dirty bottles and cases with a disinfectant before their entry to the filling hall could diminish the risk of airborne contamination of beer (Anonymous, 1988).

Isolation

Cultivation and Detection of *Pectinatus*

Many agar media and broths can be used for cultivation and isolation of *Pectinatus* strains. The most commonly used are de Man-Rogosa-Sharpe (MRS) lactobacilli broth or agar, or modified MRS medium (Schleifer et al., 1990). Peptone-yeast extract-fructose (PYF) medium (Engelmann and Weiss, 1985) intended for cultivation of *Megasphaera* species has also been used for *Pectinatus* (Satokari et al., 1997; Satokari et al., 1998; Juvonen et al., 1999). A selective medium for the isolation and differentation of *Pectinatus* spp. and *Megasphaera cerevisiae* from other brewery organisms, so-called "Selective Medium *Megasphaera*, *Pectinatus*" (SMMP) medium, was developed by Lee (1994). This new medium should replace the earlier lactate-lead acetate (LL)-medium especially designed for selective cultivation and isolation of *Pectinatus* spp. (Lee et al., 1981). The SMMP medium is beer-based and supplemented with reducing agents, various nutrients, 1% lactate as the sole carbon source, 20 ppm actidione (cycloheximide) to inhibit yeasts and 5 ppm crystal violet and 25 ppm sodium fusidate to inhibit Gram-positive bacteria. Enterobacteria are suppressed by alcohol and by hop compounds present in beer, and hence appropriate levels of ethanol must be incorporated into SMMP when nonalcoholic beer is assayed (Lee, 1994).

SMMP (Selective Medium *Megasphaera*, *Pectinatus*)

A concentrated basal medium with

Yeast extract	75 g
Bacto-peptone	75 g
DL-Lactic acid, sodium salt (60% syrup)	75 ml
Sodium thioglycollate	0.75 g
L-Cysteine HCl	0.75 g
$KH_2PO_4 \cdot 3H_2O$	7.5 g
KH_2PO_4	7.5 g
NaCl	7.5 g
$(NH_4)_2 HPO_4$	7.5 g
$NaC_2H_3O_2 \cdot 3H_2O$	7.5 g
H_2O	728.5 ml

B1 selective stock solution

Sodium fusidate[a]	0.75 g
Cycloheximide[b]	0.60 g
Crystal violet[a]	0.075–0.15 g
Absolute ethanol	100 ml

[a] Sigma or equivalent.
[b] Upjohn or equivalent.
From Lee (1994).

The American Society of Brewing Chemists recommends addition of 20-ml aliquots of basal medium and 0.5 ml of selective stock-solution to 130 ml of beer followed by incubation for 14 days at 28–30°C (Anonymous, 1997; 1998). The final concentration of crystal violet in the medium could be reduced from 5.0 to 2.5 ppm without affecting the results (Haikara, A., unpublished observations). In the original paper a freshly prepared mixture of selective agents was recommended (Lee, 1994). However, a storage time of as long as 93 days at 6°C did not abolish the selectivity of SMMP-medium (Haikara, A., unpublished observations).

More important than the media used for cultivation of *Pectinatus* and *Megasphaera* species is the maintenance of strictly anaerobic conditions for cultivation and incubation. The anaerobic glove box and the Gas Pak system provide optimum growth conditions for *Pectinatus* bacteria (Back et al., 1979; Haikara et al., 1981a; Haikara et al., 1981b; Kirchner et al., 1980; Lee et al., 1978). Using pre-reduced media and special reducing agents can enhance growth (Holdeman et al., 1977). Cultivation without an anaerobic atmosphere in broth with a minimum headspace is possible provided that the inoculum is heavy (2%) and that the oxygen is removed by boiling before use (Haikara et al., 1981a; Lee, 1984).

In quality control of beer, the presence of microorganisms is conventionally detected using membrane filtration and anaerobic incubation on an appropriate medium. Because *Pectinatus* species are sensitive to oxygen, this method is unsuitable for the detection of *Pectinatus* in beer (Haikara, 1985b). Because an anaerobic cabinet is not generally available in brewery laboratories, forcing tests or enrichment are the practical methods available in routine control of beer (European Brewery Convention, 1992a; 1992b). In forcing tests, the development of turbidity is followed in filled, closed beer bottles incubated at 30°C for 6 weeks. In the enrichment method, 15–20 ml of sterilized, concentrated MRS broth (72 g/liter) supplemented with fructose (10 g/liter) is added to the headspace of the bottle immediately after filling and capping. The bottle is reclosed with a sterile cap and incubated at 30°C for 4 weeks. After development of turbidity, the presence of *Pectinatus* and *Megasphaera* is examined microscopically. They are identified morphologically and by smell. In practice, an incubation time of 2–3 weeks is usually needed for *Pectinatus* growth, and 3–4 weeks for *Megasphaera*.

Shortening of the forcing time has been achieved by direct staining of cells on the membrane after visualization with the immunofluorescence technique or the direct epifluorescent filter technique (DEFT) with acridine orange (Haikara, 1985a; Haikara, 1985b). Direct staining has revealed contaminations one to four days before the appearance of turbidity.

Because the metabolic profiles of *Pectinatus* are distinctive (see Identification and *Megasphaera* Identification), gas chromatography also has been tested as a detection method (Haikara et al., 1981a; Haikara et al., 1981b). Detectable amounts of propionic acid were formed without visible growth of Pectinatus, one day before the turbidity appeared. Correspondingly, *n*-butyric or *n*-valeric acid produced by *Megasphaera* could be detected one day before the beer became turbid.

Winnewisser and Donhauser (1987) developed an indirect enzymatic immunotest for detection of *Pectinatus* in beer after membrane filtration. The detection limit of this assay was 50 cells/ml beer. Sample enrichment is necessary to reach the required cell density because normally there are only a few cells in one bottle of beer.

Takahashi et al. (1999) applied a new automatic MicroStar RMDS system, based on ATP bioluminescence, for rapid quantitative detection of brewery contaminants. For slow-growing *Pectinatus* bacteria, a 32-h cultivation was insufficient, but by using a high sensitivity luminous reagent for measurement, some improvement was achieved.

Cultivation and Detection of *Megasphaera*

Owing to the inability of *Megasphaera* sp. to utilize glucose, replacement of glucose with fructose in the peptone-yeast extract medium is necessary for maximal growth of *Megasphaera* species (Engelmann and Weiss, 1985). A simple PYF broth (1% peptone, 1% yeast extract, 2% fructose) also has been successfully utilized for cultivation of *Megasphaera* species (Haikara and Lounatmaa, 1987). When using SMMP medium for selective cultivation of *Megasphaera*, the change of the medium color from purple to yellow indicates the presence of *Megasphaera* bacteria (Anonymous, 1988). Because *M. cerevisiae* is a strict anaerobe, the anaerobic cultivation conditions recommended for *Pectinatus* are also valid for *Megasphaera*. Immunofluorescence, DEFT and gas chromatography have also been used as detection methods of *Megasphaera* (Haikara, 1985a; Haikara, 1985b).

Cultivation and Detection of *Zymophilus*

Strictly anaerobic conditions are required for the detection and cultivation of *Zymophilus* species. Modified MRS and MRS medium at 30°C have been used (Ziola et al., 1999; Schleifer et al., 1990). Only 14 days in a refrigerator is the maximum time for the storage of working cultures

owing to the rapid death of cells in four weeks (Seidel-Rüfer, 1990).

Ziola et al. (1999) prepared a panel of monoclonal antibodies (Mabs) for rapid detection and identification of beer-spoilage anaerobic bacteria including *Zymophilus paucivorans* and *Z. raffinosivorans*. The panel contained Mabs that were able to recognize four distinct sites (epitopes) on the bacterial peptidoglycan; hence, to facilitate antibody binding, the bacterial outer membrane must first be removed.

Identification

Pectinatus

MORPHOLOGY The most distinctive feature of *Pectinatus* species is the comb-like flagellation on only one side of the cell (Fig. 1). The number of flagella per cell depends on the cell size and condition, but is generally 1-23 (Lee, 1984). The cells of *P. cerevisiiphilus* and *P. frisingensis* are Gram-negative, slightly curved rods with rounded ends, 0.4 to 0.9 μm in diameter and 2 to 30 μm or more in length (Back et al., 1979; Haikara et al., 1981b; Lee et al., 1978; Schleifer et al., 1990). They occur singly, in pairs and only rarely in short chains. In old cultures, very elongated cells with a helical shape may be found (Fig. 2) and round cell forms are also observed. The cells are usually motile. The active, young cells form an "X" shape during movement, whereas old cells have characteristically slow snakelike movements (Lee et al., 1978). In beer the cells are thin and have a heterogeneous appearance (Seidel et al., 1979).

Colonies of *P. cerevisiiphilus* and *P. frisingensis* are circular, entire, beige to white, glistening and opaque (Haikara et al., 1981b; Schleifer et al., 1990).

ELECTRON MICROSCOPY The unique comb-like flagellar arrangement of *P. cerevisiiphilus* observed by Lee et al. (1978) using scanning electron microscopy was confirmed by other workers in negatively stained preparations (Back et al., 1979; Haikara et al., 1981b; Kirchner et al., 1980). The cell envelope structure seen in such sections revealed a multilayered cell wall typical of Gram-negative bacteria (Lee et al., 1978). The peptidoglycan layer of *Pectinatus* is very thick (30 nm), almost filling the periplasmic space of the cell envelope (Haikara et al., 1981a, Haikara et al., 1981b). The freeze-fracture technique has also been used to investigate *Pectinatus* strains (Haikara et al., 1981a; Haikara et al., 1981b). These studies have demonstrated that the cell envelope structure in different *Pectinatus* strains is similar (Haikara et al., 1981a). The

Fig. 1. Electron micrograph of *Pectinatus frisingensis* ATCC 33332.
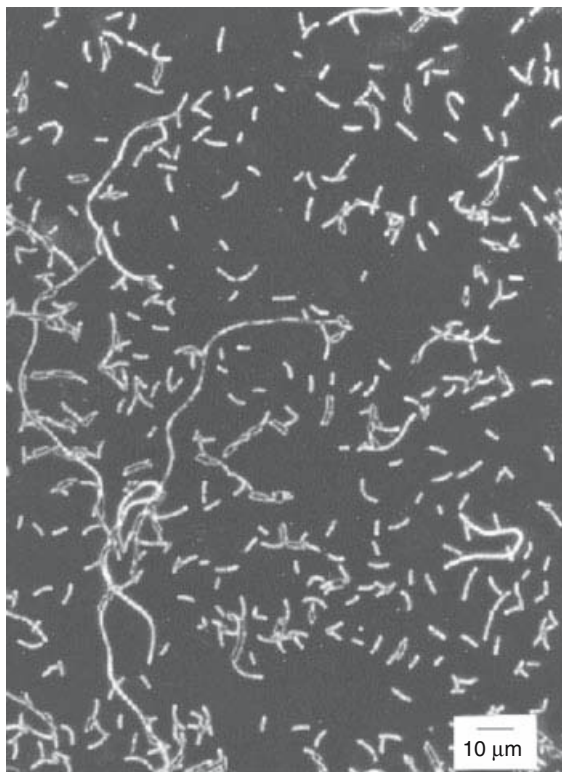
Fig. 2. Dark-field micrograph of *Pectinatus frisingensis* ATCC 33332.

thick peptidoglycan layer and the invaginations of the cytoplasmic membrane (mesosomes) are typical features of Gram-positive bacteria, whereas the outer membrane is typical of Gram-negative bacteria. The outer membrane of *Pectinatus*, however, is exceptional since it lacks permeability barrier function normally assigned to the outer membrane of Gram-negative (e.g., the enteric) bacteria (see Physiology).

CHROMATOGRAPHIC IDENTIFICATION   *Pectinatus* species are the only bacteria known to produce large quantities of propionic acid in beer, and hence gas chromatography has proved to be a useful tool for identification purposes (Back et al., 1979; Haikara et al., 1981b; Lee et al., 1978; Lee et al., 1980; Schisler et al., 1979; Membré and Tholozan, 1994; Foster and Beckler Andersen, 1999). The production of acetic acid in broth media and in beer further confirms the presence of *Pectinatus* (Foster and Beckler Andersen, 1999). The production of high levels of hydrogen sulfide (H_2S) by *Pectinatus* can also be used for identification purposes (Haikara et al., 1981a). In lager breweries, no other contaminants are able to produce H_2S. In British ale, the genus *Zymomonas* is known as an H_2S-producing spoilage organism. The simultaneous production of acetaldehyde by this bacterium, however, results in an odor of rotten apples in the ale. This is easily distinguishable, even without any instruments, from the odor of rotten eggs caused by the growth of *Pectinatus* in beer.

SEROLOGICAL CHARACTERISTICS Initial immunological investigation of the genus *Pectinatus* involving the use of polyclonal antibodies in gel immunodiffusion and immunoelectrophoresis tests revealed three serologically different groups (I, II and III) within the genus *Pectinatus* (Haikara, 1983; Haikara et al., 1981a; Haikara et al., 1981b). Groups I and III were more closely related to each other than to the more distant group II. The division of *Pectinatus* strains into two groups (I and II) was supported by differences in the cell surface protein patterns and by immunoblot analysis using surface components extracted with mild hydrochloric acid; the same antisera as were used in immunodiffusion (Haikara, 1983; Hakalehto et al., 1984; Hakalehto and Finne, 1990). Later the immunogroups I and II turned out to correspond to the designated two species of the genus, namely *P. frisingensis* and *P. cerevisiiphilus* (Schleifer et al., 1990). Previously, DNA/DNA hybridization studies had also revealed two distinct similarity groups among *Pectinatus* strains (Haikara, 1989), supporting the division of the genus *Pectinatus* into two species (see Physiology).

Gares et al. (1993) and Ziola et al. (1999) studied the reactivity of monoclonal antibodies (Mabs) with disrupted and intact *Pectinatus cerevisiiphilus* cells to detect and identify *Pectinatus* contaminations. Some Mabs reacted only with surface antigens from intact *P. cerevisiiphilus* cells and could be used in a membrane filter-based fluoroimmunoassay (MF-FIA) for detection and identification of *P. cerevisiiphilus* in contaminated beer samples (Gares et al., 1993). Using this assay, *Pectinatus* cells could be detected at concentrations as low as 2 to 4 cells in 10 ml, within less than 3 h, by trapping the bacteria on a black filter membrane and marking them with fluorescein-conjugated anti-mouse secondary antibodies. These secondary antibodies attached to the primary monoclonal antibodies that were already bound to the bacteria and thereby made them visible under a fluorescence microscope. Most of the Mabs, however, reacted only with cells from which the outer membrane had been removed, suggesting the involvement of the peptidoglycan (PG) layer in binding (Ziola et al., 1999). Because the Mabs also bound to disrupted *P. frisingensis*, *Selenomonas lacticifex*, *Zymophilus paucivorans*, *Z. raffinosivorans* and *Megasphaera cerevisiae* cells and not with other species of Gram-negative bacteria, they can be used to rapidly detect a range of anaerobic Gram-negative beer spoilage bacteria, provided that the bacterial outer membrane is first removed to allow antibody binding.

To immunologically characterize *Pectinatus* strains and to provide means of for their detection, subgrouping and identification, Hakalehto and Finne (1990) and Hakalehto et al. (1997) prepared antibodies against whole-cell preparation, flagellin protein and the N-terminal flagellar peptide of *Pectinatus* strains. An immunoluminometric analysis including a mixture of anti-peptide antibodies against antigenic sequences from *Pectinatus* (synthetic peptides) and a serum against *P. frisingensis* was developed (Hakalehto, 2000).

MOLECULAR BIOLOGICAL METHODS The polymerase chain reaction (PCR) has recently been applied for the identification and detection of *Pectinatus* species (Satokari et al., 1997; Satokari et al., 1998; Juvonen et al., 1999; Sakamoto et al., 1997; Motoyama and Ogata, 2000a). Sakamoto et al. (1997) designed highly species-specific primers for *P. frisingensis*, *P. cerevisiiphilus* and *M. cerevisiae* based on 16S rRNA sequences. The combined use of these primers and the universal primers in a single reaction guaranteed the reliability of the PCR results. Satokari et al. (1997) and Satokari et al. (1998) designed *Pectinatus* and *M. cerevisiae*-specific PCR primers from the 16S rRNA gene sequences to develop a rapid

and sensitive detection method for use in brewery quality control. Later Juvonen et al. (1999) improved the sample treatment and the sensitivity of the PCR assay by pre-enrichment. The enrichment ensured that the cells detected are still alive, thus reducing the risk of false positive results. Low levels of *Pectinatus* spp. and *M. cerevisiae* were detected after 2–4 days of pre-enrichment, depending on the strain and the alcohol content of the beer. After the pre-enrichment, the PCR analysis took less than 8 hours.

Motoyama and Ogata (2000a) constructed PCR primers from the spacer region between 16S rDNA and the 23S rDNA genes, developing a rapid and sensitive method for the detection of *P. cerevisiiphilus* and *P. frisingensis*. This method allowed the discrimination of these two closely related species because the homology of the 16S-23S rDNA intergenic spacer regions was relatively low compared to that of the 16S rDNAs (see Phylogeny; Motoyama and Ogata, 2000b).

Megasphaera

MORPHOLOGY Megasphaera cerevisiae is a Gram-negative coccus (Figs. 3 and 4). The spherical or slightly oval cells, 1.3 to 1.6 μm in diameter, occur singly or in short chains (Engelmann and Weiss, 1985). In fixed or stained preparations the cell diameter is 1.0–1.2 μm (Haikara and Lounatmaa, 1987). Cell size is one differential characteristic between the brewery species and *M. elsdenii*. In wet mounts, the diameter of *M. elsdenii* is 2.4–2.6 μm and in fixed preparations 1.2–1.9 μm, in accordance with its Latin name: big sphere (Rogosa, 1984). The ultrastructure of the cell surface of the *Megasphaera* strains isolated from beer

and of *M. elsdenii* has been reported to be very uniform (Haikara and Lounatmaa, 1987). *Megasphaera cerevisiae* forms whitish, smooth, opaque, flat and shiny colonies, 2–5 μm in diameter (Engelmann and Weiss, 1985; Weiss et al., 1979).

CHROMATOGRAPHIC IDENTIFICATION The distinctive metabolic profile of *Megasphaera cerevisiae* (Haikara, 1985a; Weiss et al., 1979; Foster and Beckler Andersen, 1999) provides a useful tool for its identification. This profile includes propionic, isobutyric, isovaleric, valeric and caproic acids. The gas chromatographic detection of these acids in brewery samples can be regarded as proof of the presence of *M. cerevisiae* (Foster and Beckler Andersen, 1999). The identification can be complemented with detection of H_2S produced by *Megasphaera*.

SEROLOGICAL CHARACTERISTICS Hakalehto (2000) produced anti-peptide antibodies against *Megasphaera* surface protein (40–50 kDa) and found crossreactivity in the immunoblots between *Pectinatus* and *Megasphaera* protein bands. Similarly the antibodies produced with the N-terminal peptide of the *Pectinatus* 16-kDa surface protein exhibited specific activity against the *Pectinatus* 51-kDa surface protein band from immunoblots and against the *Megasphaera* surface protein band of slightly smaller molecular weight.

Using *P. cerevisiiphilus* cells Ziola et al. (1999) succeeded in producing peptidoglycan (PG)-specific Mabs that reacted not only with *Pectinatus*, *Zymophilus* and *Selenomonas* species, but also with *Megasphaera cerevisiae*. Although the
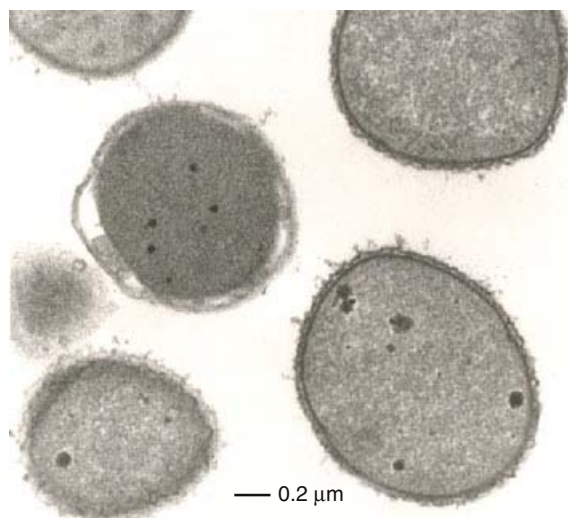
Fig. 3. Electron micrograph of *Megasphaera cerevisiae* VTT E-84195.
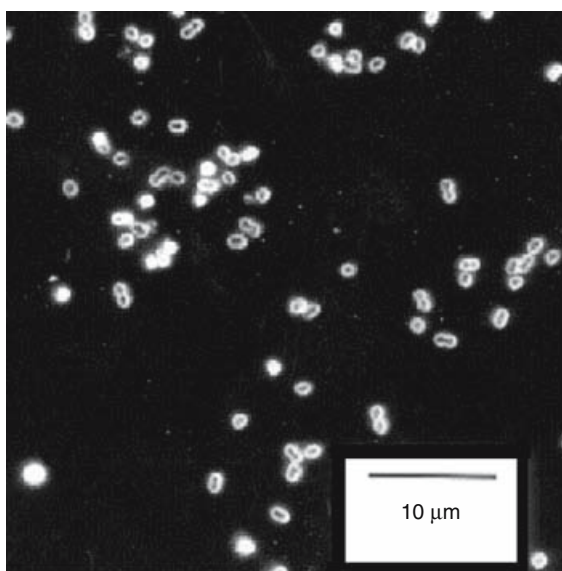
Fig. 4. Dark-field micrograph of *Megasphaera cerevisiae* VTT E-84195.

binding of PG epitopes of *M. cerevisiae* was weak, the PG Mabs also could potentially be used for rapid detection of Megasphaera. For selective detection and identification of *Megasphaera cerevisiae*, bacterial surface-reactive Mabs corresponding to the three different serogroups were produced and used in a fluoro-immunoassay format (Ziola et al., 2000).

MOLECULAR BIOLOGICAL METHODS PCR-based methods have recently been developed for the detection and identification of *M. cerevisiae* (Satokari et al., 1998; Sakamoto et al., 1997; Juvonen et al., 1999). Satokari et al. (1998) developed a method in which amplified PCR products were analyzed by the colorimetric microplate hybridization method. A biotinylated PCR product was captured by streptavidin and hybridized with a digoxigenin-labelled oligonucleotide probe. In the final step an enzyme-linked antibody and a colorimetric reaction were utilized.

Juvonen et al. (1999) combined a pre-enrichment with the PCR assay and managed to detect low levels of *M. cerevisiae* cells (after 2–3 days of enrichment) by gel electrophoretic analysis of the PCR products. The universal primers were used as an internal positive control in the PCR method, based on 16S rDNA sequences, and applied to species-specific detection of *M. cerevisiae* (Sakamoto et al., 1997).

Zymophilus

MORPHOLOGY Schleifer et al. (1990) described *Zymophilus raffinosivorans* as straight to slightly curved, motile, Gram-negative rods (0.7 to 0.9 by 3 to 15 μm in size) with rounded ends, occurring predominantly singly and sometimes in pairs or short chains. Colonies on modified MRS agar medium are circular, smooth, opaque, slightly yellow, and 1 to 2 μm in diameter after 3 days at 30°C.

Zymophilus paucivorans cells are motile, curved, helical or crescent-shaped, Gram-negative rods (0.8 to 1.0 by 5 to 30 μm) with rounded ends (Schleifer et al., 1990). They occur singly or in pairs or short chains. Colonies on modified MRS agar medium are circular, smooth, slightly yellow and 1 to 2 μm in diameter after 3 days at 30°C.

Preservation

Working cultures of *Pectinatus*, *Megasphaera* and *Zymophilus* can be maintained by subculturing (30°C, 2–3 days) at least every two weeks in PYF broth (0.5% peptone, 0.5% tryptone, 1% yeast extract, 0.5% fructose, 0.2% Na₂HPO₄,

0.1% Tween 80, and 0.05% cysteine hydrochloride added as a reducing agent). The *Selenomonas* and *Zymophilus* strains isolated from pitching yeast lose their viability rapidly (Seidel-Rüfer, 1990; Haikara, 1991), and subculturing every week is necessary. After incubation, the cultures are stored at 4°C in anaerobic jars. The working cultures can also be maintained in 5% dimethyl sulfoxide in plastic screw-cap ampoules frozen at −70°C.

For long-term preservation conventional freeze-drying methods using 20% skim milk as protective agent as well as freezing in drinking straws in a −150°C deep freezer or in liquid nitrogen at −196°C using 5% dimethyl sulfoxide as protective agent have been used. The polypropylene straws are packed after filling (0.1 ml) into 2-ml plastic screw-cap ampoules (e.g., Nunc) and placed directly into liquid nitrogen (Suihko and Haikara, 1990).

Physiology

Physiological and Biochemical Properties of *Pectinatus*

Pectinatus species are catalase- and cytochrome oxidase-negative, and do not produce indole, liquify gelatin, reduce nitrate, or hydrolyze arginine. The Voges-Proskauer test is positive for all strains (Back et al., 1979; Haikara et al., 1981b; Kirchner et al., 1980; Lee et al., 1978). Only the urease test has given variable results for different strains (Back et al., 1979; Haikara et al., 1981b).

Pectinatus species grow at temperatures between 15 and 40°C, the optimum being 30 to 32°C (Back et al., 1979; Haikara et al., 1981b; Kirchner et al., 1980; Lee et al., 1978). Chowdhury et al. (1995) studied the effect of temperature on their survival and oxygen resistance. The survival was better at lower temperatures (8°C, 15°C) than at 32°C. Temperature also inversely influenced the oxygen resistance of *Pectinatus*. The time of decimal reduction (an inactivation kinetic parameter used to indicate rate of microbial reduction) decreased by 6.7 fold when the temperature decreased from 32°C to 8°C. The oxygen resistance of *P. frisingensis* was higher than that of *P. cerevisiiphilus*, which has also previously been observed (Haikara, 1985b). In wort, dissolved oxygen levels below 0.96 mg/liter made the growth of *Pectinatus* possible (Chowdhury et al., 1995).

Pectinatus strains grow well in the pH range 4.5–8.5 (Back et al., 1979; Haikara et al., 1981b), the optimum pH being between 6.0 and 7.0 (Kirchner et al., 1980; Takahashi, 1983). However, rather good growth occurs even in the pH

range 4.0 to 4.5 (Kirchner et al., 1980). The acid tolerance of *Pectinatus* was also verified in growth tests in beer (Haikara, 1984). The normal pH of Finnish beer (average 4.4) did not restrict the growth of *Pectinatus* sp. Some retardation of growth was detected at pH 4.1. In German Alt beer (pH 4.0), the growth of *Pectinatus* was considerably reduced but still not totally prevented (Kirchner et al., 1980; Seidel et al., 1979). In the German beer type Berliner Weisse, with a pH of 3.1, no growth occurred (Kirchner et al., 1980).

A number of studies on *Pectinatus* growth in the culture medium as a function of temperature, pH and ethanol concentration have been performed (Tholozan et al., 1996; Tholozan et al., 1997; Chihib and Tholozan, 1999a; Chihib et al., 1999b) including modeling of growth and metabolite production (Membré and Tholozan, 1994; Watier et al., 1996b). In general, the parameters derive from those encountered in beer production, including rapid temperature changes in pasteurization and cooling before bottling. Whereas ethanol concentrations of 1.7 M (8%) totally inhibit the growth of both *Pectinatus* species (Tholozan et al., 1996; Watier et al., 1996b), *P. frisingensis* appears to be less sensitive than *P. cerevisiiphilus*, which does not tolerate ethanol at 1.3 M (Tholozan et al., 1996; Tholozan et al., 1997). Similarly, *P. frisingensis* copes better with low pH and is able to grow over a wider pH range than *P. cerevisiiphilus*; for the latter, pH 6.2 is optimal for growth and biomass production, whereas for the former, pH of approximately 4.9 is optimal for growth and biomass production in glucose concentrations below 20 mM (Tholozan et al., 1997). In accord with its higher acid tolerance, *P. frisingensis* is able to maintain a higher intracellular pH than the external pH even at pH 4.5 (Chihib and Tholozan, 1999a). A cooling treatment from 30°C to 2°C does disturb the cellular homeostasis of P. frisingensis but this effect is only temporary, as the bacterium is able to restore homeostasis under warm conditions and in the presence of a carbon source (Chihib and Tholozan, 1999a). The physiological characteristics of *P. frisingensis* as compared to *P. cerevisiiphilus* may suggest that the former species is the prevailing one in beer spoilage.

Although the structure of *Pectinatus* lipid A, with a large degree of phosphate substitution by 4-amino-4-deoxyarabinose, would suggest that these bacteria are resistant to cationic agents such as polymyxin B, the opposite turns out to be true (Helander et al., 1994). This surprising result is explained by findings indicating that the outer membrane of *Pectinatus* does not form an effective barrier: the above study also demonstrated a high sensitivity of *Pectinatus* strains to vancomycin and bacitracin, which are rather large molecules (M_r, 1449 and 1411, respectively)

unable to penetrate a normal outer membrane in most bacteria (Vaara, 1993). Furthermore, *P. frisingensis* was shown to be sensitive to nisin, which is a cationic peptide acting lethally on the bacterial cytoplasmic membrane, but is unable to penetrate an intact outer membrane (Chihib et al., 1999b). Gram-negative bacteria are normally insensitive to nisin action (Helander et al., 1997). Supported by electron microscopic observations that Pectinatus strains have an abnormally wrinkled outer membrane (Haikara et al., 1981b; Lee et al., 1978), the conclusion has been drawn that the outer membrane of *Pectinatus* has cracks that permit the entry of various external compounds.

Metabolism

Clear differences exist in the utilization of carbon sources by the two *Pectinatus* species (Schleifer et al., 1990; Haikara, 1991; Table 2). In contrast to other brewery contaminants, *Pectinatus* strains are incapable of utilizing maltose, the main carbohydrate of wort. On the other hand they can utilize lactate. Thus the presence of lactic acid bacteria in the brewery environment can facilitate the growth of *Pectinatus*.

The main metabolic products of *Pectinatus* species from glucose are propionic, acetic, succinic and lactic acids and acetoin (Back et al., 1979; Haikara et al., 1981b; Lee et al., 1978; Tholozan et al., 1994; Table 1). The relative amounts of metabolites are dependent on the substrate utilized by *Pectinatus* (Back et al., 1979; Lee et al., 1978). However, no differences in this respect were found between different strains (Haikara et al., 1981b). The most abundant acid produced by *Pectinatus* is propionic acid. The amount found in contaminated beer is rather high, often more than 1,000 mg/liter (Haikara et al., 1981a; Takahashi, 1983).

The organic acids produced by *Pectinatus* and the low pH were obviously the main reasons for the growth inhibition and alcohol production of *Saccharomyces cerevisiae* in mixed fermentations above 15°C (Chowdhury et al., 1997). No effect was found in typical lager beer fermentations around 8–15°C.

Haikara et al. (Haikara et al., 1981a; Haikara et al., 1981b) and Tholozan et al. (1994) studied the mechanism of propionic acid synthesis by *Pectinatus*. *Pectinatus* bacteria use the same pathway for production of propionic acid as do propionibacteria. In this pathway, succinate oxidoreductase catalyzes the reduction of fumarate to succinate (Hettinga and Reinhold, 1972). Malonate is a specific competitive inhibitor of this enzyme and can thus inhibit the production of propionic acid, leading to a corresponding increase in acetic acid production. This was shown to occur in different *Pectinatus* strains and

also in *Propionibacterium* species (Haikara et al., 1981a; Haikara et al., 1981b). This observation was taxonomically significant because *Bacteroides* species are known to use the acrylate pathway for propionate synthesis (Wallnöfer and Baldwin, 1967). Later Tholozan et al. (1994) confirmed propionate production by *P. frisingensis* through the succinate pathway by measuring key enzyme activities and by studying the utilization of specially labeled metabolic precursors. The authors also proposed a tentative metabolic pathway to the main fermentation products of *P. frisingensis*.

A very specific feature of the metabolism of *Pectinatus* sp. is the production of organic sulfur compounds (Haikara et al., 1981a). In addition to H_2S detected by Lee et al. (1980), production of methyl mercaptan and dimethyl trisulfide has been observed. Gas chromatographic analysis did not reveal the formation of dimethyl disulfide or thiolacetate.

Physiological and Biochemical Properties of *Megasphaera*

Megasphaera elsdenii has been used as a reference species in the physiological and biochemical tests of the beer isolate (Weiss et al., 1979; Haikara and Lounatmaa, 1987). The optimum temperature for *Megasphaera* beer isolates is 30–37°C and for *M. elsdenii* 37–42°C. *Megasphaera elsdenii* does not grow at room temperature but the brewery isolates can grow even at 15°C. A slightly lower optimum temperature of 28°C has been reported for the type strain *M. cerevisiae* (Engelmann and Weiss, 1985).

Megasphaera sp. is more sensitive to low pH than *Pectinatus* sp., especially in beer. Increase in the pH of beer from 4.1 to 4.7 was found to accelerate the growth of *Megasphaera* sp. (Haikara and Lounatmaa, 1987). No growth occurs at pH 4.1. Seidel et al. (1979) also reported the growth of *Megasphaera* sp. in beer of pH from 4.38 to 4.57, but not at pH 4.0.

In addition to low pH, alcohol is the most important factor inhibiting the growth of *Megasphaera* in beer. Very small amounts of ethanol retard the growth of *Megasphaera* sp. (Haikara and Lounatmaa, 1987). The dependence of the rate of spoilage on the alcohol content was also demonstrated in experiments in which growth of *Megasphaera* sp. was monitored in commercial beers with different alcohol contents (Haikara, 1991). Growth did not occur in beer with an alcohol content of 4.3% w/v. Correspondingly, no growth was observed in German beer with alcohol contents over 5.5 and 6.5% (v/v; Back, 1981; Seidel et al., 1979).

Utilization of different carbon sources by *M. cerevisiae* is presented in Table 2. The carbohydrate spectrum of *M. cerevisiae* is very narrow compared with that of *M. elsdenii*. The brewery isolates do not utilize glucose or maltose, but like *M. elsdenii*, they can grow in sugar-free medium although growth is very poor (Engelmann and Weiss, 1985; Weiss et al., 1979). With respect to the fermentation of carbohydrates, the brewery isolates of *Megasphaera* form a very uniform group. Like *Pectinatus*, they can utilize lactate and, hence, lactic acid bacteria could in practice facilitate their growth.

Metabolic end products of *Megasphaera* spp. are *iso-* and *n*-butyric acid, *iso-* and *n*-valeric acid, acetic, propionic and caproic acids (Engelmann and Weiss, 1985; Haikara, 1985a; Weiss et al., 1979; Table 1). The predominant end product from lactate is *n*-valeric acid. In beer, the main metabolic product is *n*-butyric acid (Haikara and Lounatmaa, 1987). The production of caproic acid is a typical feature of this genus. All *Megasphaera* species produce H_2S (Engelmann and Weiss, 1985; Weiss et al., 1979). Owing to the mixture of various fatty acids and H_2S, the flavor of contaminated beer is particularly unpleasant.

All *Megasphaera* strains isolated from beer are catalase-, benzidine- and Voges-Proskauer-negative; do not hydrolyze urea, arginine, gelatin or esculin; do not reduce nitrate; and do not form indole (Weiss et al., 1979).

Exceptional in that it exhibits increased heat resistance at low pH in wort, *M. cerevisiae* DSM 20461 was found to be more resistant to heat (on the basis of D–50 values) at pH 4 than at pH 5.2 or 6.0 (Watier et al., 1996a).

Physiological and Biochemical Properties of *Zymophilus*

Zymophilus species are obligately anaerobic, motile, nonsporeforming, Gram-negative rods. The optimum temperature for growth is 30°C (see Isolation). Both species ferment glucose to acetic and propionic acids (Table 1). Trace amounts of lactic acid are produced by *Z. paucivorans*. Physiological properties and G+C contents of both *Zymophilus* species are listed in Table 2 (Schleifer et al., 1990). The data is based on ten *Z. raffinosivorans* strains isolated from pitching yeast and brewery waste and on four *Z. paucivorans* strains isolated from pitching yeast. *Zymophilus* species are not only morphologically quite different, but they also differ in the utilization of xylose, rhamnose, xylitol, raffinose, melibiose and inositol (Table 2).

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CHAPTER 1.3.3

The Genus *Selenomonas*

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Species of *Selenomonas* are defined as anaerobic, Gram-negative, curved or crescent-shaped rods that are motile by means of a tuft of flagella originating from the inner curvature of the cell. Selenomonads have been isolated from the rumen and ceca of mammals, and the human oral cavity. Depending upon the health or diet of the host, these bacteria can constitute a significant proportion of the total microbial population. In general, selenomonads are obligately saccharolytic, although some strains ferment lactate or amino acids. It has been suggested that the role of ruminal and intestinal selenomonads involves the fermentation of soluble sugars and lactate in their natural environments. Oral selenomonads may play a role in the pathogenesis of periodontal disease in humans. The first classification of selenomonads was by Miller (1887), who designated strains from the human mouth as *Spirillum sputigenum*, but the present classification system places these organisms in the genus *Selenomonas* as *S. sputigena* (Bryant, 1984; Johnson et al., 1985). As a result of recent findings, however, at least six species of oral selenomonad have been recognized (Moore et al., 1987). Phylogenetic studies based on 16S ribosomal RNA sequence analysis have shown that species of *Selenomonas* are closely related to the bacteria *Centipeda periodontii, Pectinatus cerevisiiphilus*, and *Sporomusa paucivorans*. The selenomonads are more distantly related to anaerobic Gram-negative cocci of the genera *Veillonella* and *Megasphaera*. Selenomonads, *Veillonella* and related bacteria comprise a phylogenetic grouping which is more closely related to Gram-positive bacteria than to typical Gram-negative bacteria.

Habitats

Selenomonads have been observed in and isolated primarily from the rumen, the human mouth, and the cecum of mammals (Table 1). Gram-negative organisms having the cell morphology and flagellar arrangement typical for selenomonads have also been observed in river water (Leifson, 1960), and recently selenomonads have been isolated from anaerobic sewage sludge (Nanninga et al., 1987) and bog water (Harborth and Hanert, 1982). Selenomonads were probably first observed by Antonie van Leeuwenhoek in gingival scrapings from the human mouth (Dobell, 1960). Traditionally, speciation within *Selenomonas* has been based upon the habitat from which the strain was isolated (Buchanan and Gibbons, 1974; Lessel and Breed, 1954). Although helpful, this criterion has been largely supplanted by more adequate cytological, biochemical, and molecular criteria.

Selenomonads appear to be part of the normal indigenous microflora of human gingival crevices and are often more abundant in those persons having clinically detectable gingivitis or peridontal disease. Oral selenomonads may play a role in peridontal disease, inasmuch as lipopolysaccharide purified from these bacterial species has been shown to possess several endotoxic properties in mice (Kurimoto et al., 1986). Some occurrences of *S. sputigena* and other selenomonads in human septicemia have been reported (MacCarthy and Carlson, 1981; Pomeroy et al., 1987).

Selenomonads isolated from the rumen are usually shown to be strains of *S. ruminantium*. These organisms are routinely observed and isolated from rumen contents of cows and sheep. (Bryant, 1956; Hobson and Mann, 1961; Prins, 1971). In general selenomonads are more numerous in animals fed rations such as grains, which contain rapidly fermentable carbohydrates, than they are in animals fed silage or straw (Caldwell and Bryant, 1966). In addition to the fermentation of soluble carbohydrates, rumen selenomonads have other important roles in the rumen. These organisms are among the most important members of the glycerol-fermenting species of ruminal bacteria in sheep (Hobson and Mann, 1961) and in cattle (Bryant, 1956). While lactate is fermented by only a few species of ruminal bacteria, many ruminal selenomonad strains ferment lactate. Often, these strains are designated as *S. ruminantium* subspecies *lactilytica* and are almost phylogenetically identical to *S. ruminantium* (Fig. 1). Lower ruminal pH and increased lactate formation resulting from bloat or high-grain feeding, can lead to a substantial increase of ruminal selenomonads. Urea present in saliva and feedstuffs is degraded in the rumen by urease to form ammonia, a

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This chapter was taken unchanged from the second edition.

Table 1. Strains, sources, and DNA base composition of *Selenomonas* species.

Species	Natural source	Strain[a]	GC content (mol%)
S. acidaminophilia	Sewage sludge	DSM 3853	48.0
S. ruminantium	Rumen	ATCC 12561	49.0
S. ruminantium	Rumen	HD1	50.5
S. ruminantium	Rumen	HD4	53.5
S. ruminantium	Bog water	—	51.6
S. palpitans	Guinea pig cecum	—	—
S. artemidis	Gingival crevice	ATCC 43528	58.0
S. noxia	Gingival crevice	ATCC 43542	57.0
S. flueggei	Gingival crevice	ATCC 43531	56.0
S. infelix	Gingival crevice	ATCC 43532	58.0
S. sputigena	Gingival crevice	ATCC 35185	57.0
S. dianae	Gingival crevice	ATCC 43527	53.0

ATTC = American Type Culture Collection, Rockville, MD, U.S.A.; DSM = Deutsche Sammlung von Mikroorganisms, Göttingen, FRG; Strains HD1 and HD4 from M. P. Bryant.
Data from Nanninga et al. 1987; Kingsley and Hoeniger (1973); and Moore et al. 1987.
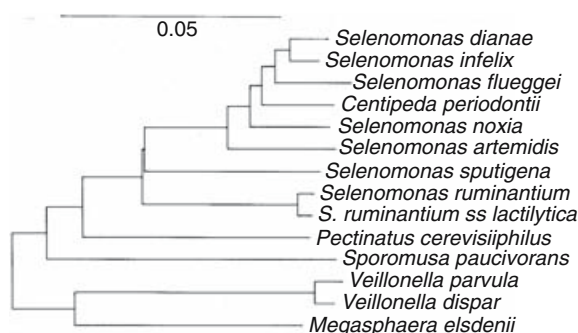
Fig. 1. Phylogenetic tree for the selenomonads and related bacteria. The scale indicates a 5% difference in nucleotide sequence, as determined by taking the sum of all branch lengths connecting two species. (Sequences for *S. paucivorans* and *M. elsdenii* courtesy of C. R. Woese.)

major nitrogen source for growth of many rumen microorganisms. *S. ruminantium* probably contributes to the hydrolysis of urea because urease-producing strains are commonly isolated (Wozny et al., 1977).

Selenomonad strains that have been phenotypically classified as *S. ruminantium* constitute a significant part of the intestinal microbial populations of swine. Approximately 21% of the total bacterial isolates from cecal contents of healthy swine were strains of *S. ruminantium* (Robinson et al., 1981). *S. ruminantium* strains were found to represent about 5% and 15% of the total bacteria associated with the epithelial tissue of the colon in healthy and dysentery-infected swine, respectively (Robinson et al., 1984). Although this last study did not find selenomonad strains in luminal contents of the colon, these organisms have been detected in swine feces (Salanitro et al., 1977). Presumably, a functional role for selenomonads in the swine intestine involves fermentation of soluble sugars as occurs with these organisms in the rumen. The dominance of *S. ruminantium* strains in the swine intestine may

be related to the availability of lactate. The swine of the small intestine can contain high levels of lactate (15 to 20 mM) and lactate levels decrease almost 10-fold upon reaching the cecum (Imoto and Namioka, 1978). In this regard, all of the selenomonad strains isolated from cecal contents have been lactate-fermenting strains, namely *S. ruminantium* subsp. *lactilytica* (Robinson et al., 1981). Lactate may also be available to *Selenomonas* strains that adhere to the colon wall since other adherent bacteria include lactate-producing species such as *Lactobacillus and Enterococcus* (Robinson et al., 1984).

Selenomonas species have frequently been observed and isolated from the cecum of a number of small rodents. The study by Ogimoto (1972) indicated that about 5% of the total bacterial isolates from rat cecal contents were selenomonads. All their rat strains could ferment any one of a number of sugars, including cellobiose; propionate was the major fermentation acid. With freeze-fracture electron microscopic techniques, selenomonads could be seen in the rat cecum submucosa. Gram-negative organisms that have the cell morphology and flagellar arrangement typical for selenomonads have been observed in cecal contents of squirrels. These organisms were enumerated and isolated from cecal contents of the 13-lined ground squirrel (*Citellus tridecemlineatus*) and shown to be as high as 1×10^9 to 1×10^{10} cells per gram of cecal contents (Barnes and Burton, 1970). In addition, it was shown that these selenomonads constituted 18% or more of the total viable cells of the cecal contents of both active and hibernating squirrels. All selenomonad strains from squirrels could ferment glucose and some strains could ferment starch, but further biochemical characteristics of these strains have not been determined. Many investigators have observed selenomonads in the cecal contents of guinea pigs (Kingsley and Hoeniger, 1973; Robinow,

1954). These organisms have been designated as *S. palpitans* (Simons, 1922), but since the organisms have not been isolated or grown in pure culture, this classification is questionable. However, electron micrographs of "*S. palpitans*" indicate it is a selenomonad which differs in some respects in cell morphology from *S. ruminantium* (Kingsley and Hoeniger, 1973).

The isolation of selenomonads from nonmammalian environments has been reported only on one occasion; using lactate and sulfate enrichment cultures, a selenomonad strain was isolated from ditch water from a bog habitat (Harborth and Hanert, 1982). This strain was phenotypically similar to *S. ruminantium* except it has a lower optimal growth temperature (25°C) and produces catalase. The authors suggested it be considered *S. ruminantium* subspecies *psychrocataligenes*. Selenomonad strain DSM 3853 (strain DKglu16) was isolated from glutamate-plus aspartate-limited chemostat cultures inoculated with anaerobic sewage sludge (Nanninga et al., 1987). Because this strain fermented only amino acids and differed in many other ways from other *Selenomonas* species, it was named *S. acidaminophila*. These studies suggest that selenomonads may exist in a number of natural, anaerobic habitats.

Isolation

Selective Enrichment and Isolation

Enrichment and selection procedures have been effectively developed only for the isolation of selenomonad strains from ruminal contents. Ruminal organisms can be selectively isolated using SS medium that has mannitol as the only added carbohydrate (Tiwari et al., 1969). The selective factors of SS medium are: 1) the use of mannitol as the main energy source, (since few species of ruminal bacteria can ferment this sugar); 2) the pH of the medium is 6.0, which does not affect selenomonad growth, but inhibits growth of many other species; and 3) the medium contains no branched-chain volatile fatty acids or heme, and either or both of these compounds are required for the growth of many other ruminal bacteria. Substitution of glycerol or lactate for mannitol in SS medium might allow for preferential isolation of selenomonad strains commonly designated as *S. ruminantium* subspecies *lactilytica*.

Selective Medium (SS) for Isolation of *Selenomonas ruminantium* (Modified from Tiwari et al., 1969)

Mannitol	0.2 g
Trypticase	0.5 g
Yeast extract	0.1 g
Sodium acetate	0.1 g
FeSO$_4 \cdot$ 7H$_2$O	0.1 g

Mineral S solution (see below)	4.1 ml
n-Valeric acid	0.05 ml
Distilled water	92.5 ml
L-Cysteine · HCl solution (2.5%) (see below)	1.0 ml
Sodium carbonate solution (8%) (see below)	2.5 ml

The L-cysteine solution is prepared with oxygen-free distilled water under a nitrogen-gas phase. Both the sodium carbonate solution and final SS medium are prepared and equilibrated under a carbon dioxide gas phase. The L-cysteine and sodium carbonate solutions are autoclaved separately and added to the cooled medium. The composition of the mineral S solution is: monobasic potassium phosphate, 12.0 g; ammonium sulfate, 6 g; sodium chloride, 12.0 g; magnesium sulfate heptahydrate, 2.5 g; calcium chloride dihydrate, 1.6 g; and distilled water to a final volume of one liter. This solution is stable when stored at 5°C.

In regard to other species of *Selenomonas*, some enrichments for these organisms appear possible from samples of anaerobic sludge, bog water, or highly eutrophic waters. With such samples, the use of glutamate or aspartate as the main carbon source may lead to enrichment of *S. acidaminophila* strains (reference is not an exact match Nanninga et al., 1982), whereas use of lactate may result in enrichment of *S. ruminantium* strains (Harborth and Hanert, 1982).

MacDonald and Madlener (1957) examined several methods for isolating oral selenomonads. These authors found that complex media containing sodium lauryl sulfate (0.01%) or sodium oleate (0.15%) in addition to sheep serum (10%) would inhibit the growth of many microorganisms present in gingival scrapings, but would not affect selenomonad growth. Nonselective media, such as commercially available blood agar, can sometimes be used for the isolation of oral selenomonads. In certain subgingival sites that possess clinical signs of periodontitis, oral selenomonads can represent 10–30% of the total bacterial population (S. S. Socransky, personal communication). Appropriate dilutions of clinical samples are plated onto agar media and incubated under an atmosphere of 80% N$_2$/10% CO$_2$/10% H$_2$. Identification of these motile bacteria is initially based upon colony morphology. Since cells are able to migrate through solid medium with lowered concentrations of agar, colonies with a spreading, fuzzy morphology form. Some species of *Selenomonas* or *Centipeda* form a hazy zone of growth on the surface of the agar medium (Lai et al., 1983).

Cultivation Media

The nutrient requirements of *S. ruminantium* strains are rather simple, and most, if not all, strains can be grown anaerobically in chemically defined media containing glucose, minerals, B vitamins, ammonia, sulfide, and a volatile fatty acid (usually n-valerate), under a carbon dioxide

atmosphere (Bryant and Robinson, 1962; Kanegasaki and Takahashi, 1967; John et al., 1974; Tiwari et al., 1969). More complex media containing yeast extract and trypticase, such as those used for *Succinivibrio dextrinosolvens* (see The Family Succiaivibrionaceae in Volume 3), will support growth of *S. ruminantium* strains. These complex media probably can support growth of *S. acidaminophila*, but glutamate, aspartate, pyruvate, or lactate would be needed as the energy source (Nanninga et al., 1987). For *S. ruminantium*, sulfide or cysteine serve as sole sulfur sources. Substances that can serve as sole nitrogen sources include ammonia, urea, certain single amino acids (cysteine, serine, threonine, aspartate, histidine, glutamate, and valine), or the purines adenine and uric acid, Some strains utilize urea or ammonia as a sole nitrogen source, but many strains do not. When lactate is the energy source, biotin and *p*-aminobenzoic acid satisfy the vitamin requirements; aspartate, malate, or fumarate are required for growth and aromatic amino acids may be stimulatory to growth (Linehan et al., 1978).

The nutritional features of most human oral species of *Selenomonas* have not been studied in detail, as has been done with *S. ruminantium*. Most of these species have been isolated on very complex media, such as brain-heart infusion agar or on this medium supplemented with 5% rabbit blood or serum. Rich media such as peptone-yeast extract-glucose often support growth of many of these species (Moore et al., 1987).

Preservation of Cultures

Most species can be maintained for long periods by storing cultures in liquid nitrogen or ultracold freezers (Hespell and Canale-Parola, 1970). Depending upon the strain, preservation by lyophilization under anaerobic conditions may be possible. Alternatively, short-term storage (6 to 15 months) may be possible by placing glycerol-containing cultures in normal (−20°C) freezers (Teather, 1982).

Identification

Phenotypic Properties

Quite often, newly isolated bacterial strains can be identified as *Selenomonas* based on the characteristic cell shape and tumbling motility of these species. All species are obligately anaerobic, motile, nonsporeforming, Gram-negative rods. The cells are usually curved or cresent-shaped and have a tuft of flagella that originates from the concave side of the cell, as revealed by Leifson flagella staining or by electron micros-

copy. Strains of the related genus *Pectinatus* have a similar curved-cell morphology, but the flagella are arranged linearly along the entire length of the concave side of the cell (Lee et al., 1978). *Centipeda* strains are Gram-negative, curved or helical rods. However, the flagella *Centibeda* are inserted in a line or stop that spirals around the cell, resulting in bundles that arise from both cell sides, giving a centipede-like appearance to the cell (Lai et al., 1983). A major characteristic of all known *Selenomonas* species is that they produce both acetate and propionate as major fermentation acids. Many strains also form small amounts of lactate and/or succinate (Table 2).

Species of *Selenomonas* have varying types of colony morphologies and cell sizes. Most strains of *S. ruminantium* produce large colonies (3- to 6-mm diameter) that are smooth, entire, slightly convex, and light tan to white in color. Often, these colonies have a gray to black appearance (due to hydrogen sulfide production) starting in the center of the colony. The cells are usually 1 μm by 2.0 to 4.0 μm long, but *S. ruminantium* subspecies *lactilytica* strains can be 2.0–3.0 μm by 5.0–10.0 μm long. Many *S. ruminantium* strains have carbohydrate granules in the cytoplasm, and cells may be strongly iodophilic (Prins, 1971) but no capsular material is present.

With newly isolated strains, *S. sputigena* colonies on blood-agar media are generally small (0.5 to 1.2 mm in diameter), smooth, convex, and gray to gray-yellow in color. Larger colonies tend to have an irregular edge and translucent appearance. Most of the other oral selenomonad species form minute colonies (0.5–1.0 mm in diameter) that are shiny, smooth, and colorless to white. A spreading growth over the entire plate is not uncommon for several species. The cell sizes of most oral selenomonad species range from about 1.0–1.4 μm wide by 3.0–5.5 μm long.

Species of *Selenomonas*, *Pectinatus*, and *Centipeda* can be differentiated from one another on the basis of a number of phenotypic traits (see Table 2). *C. periodontii* is the only species listed that has a bilateral flagella arrangement and that does not form acetate as a major fermentation acid, although trace amounts of acetate can be made by some strains. *P. cerivisiiphilus* and *S. ruminantium* are the only listed species to produce hydrogen sulfide. *P. cerivisiiphilus* differs from *S. ruminantium* by its linear array of flagella and its inabilities to ferment sucrose and hydrolyze esculin (Lee et al., 1978). *S. acidaminophila* differs from all other listed species by its inability to ferment sugars, by using only lactate, pyruvate, glutamate, and aspartate, and by being able to hydrolyze gelatin. *S. ruminantium* is the only *Selenomonas* species capable of fermenting cellobiose. *S. infelix* and *S. dianae* are the only oral *Selenomonas* species capable of esculin

Table 2. Characteristics that differentiate species of *Selenomonas*, *Pectinatus*, and *Centipeda*.[a]

Characteristic	S. acidaminophila[b]	S. ruminantium[c]	S. artemidis[d]	S. noxia[d]	S. flueggei[d]	S. infelix[d]	S. sputigena[d,f]	S. dianae[d]	P. cerevisiiphilus[e]	C. periodontii[f]
Acid from:										
Lactate	+	V	–	–	–	–	NK	–	+	+
Pyruvate	+	–	+	–	+	+	NK	–	NK	NK
Glutamate	+	–	NK	NK	NK	NK	NK	NK	NK	NK
Sucrose	–	+	+	–	+	+	+	+	–	+
Cellobiose	–	+	–	–	–	–	–	–	+	V
Mannitol	–	+	+	–	+	+	+	+	+	+
Lactose	–	V	–	–	+	+	+	+	–	+
Trehalose	–	V	–	–	–	–	–	+	–	V
Esculin hydrolysis	–	+	–	–	–	+	–	+	–	V
Hydrogen sulfide production	–	+	–	–	–	–	–	+	+	–
Nitrate reduction	–	V	+	–	+	+	v	+	–	+
Gelatin hydrolysis	+	–	–	–	+	–	–	–	–	–
Fermentation acids	A,P,s	A,P	A,P,L,s	A,P	A,P,l	A,Pl	A,P,l,s	A,P,L	A,P,l,s	a,P,l,s

[a]Positive = +; negative = –; variable = V; not known = NK. Major acid = upper case; minor acid = lower case: acetate = A; propionate = P; lactate = L; succinate = S.
[b]Data from Nanninga et al. (1987).
[c]Data from Bryant (1956).
[d]Data from Moore et al. (1987).
[e]Data from Lee et al. (1978).
[f]Data from Lai et al. (1983).

hydrolysis, and these two species can be separated on the basis of acid production on trehalose. With respect to the esculin-negative oral *Selenomonas* species, *S. flueggei* and *S. sputigena* are both positive for acid from lactose and can be separated on the basis of acid from mannitol, whereas *S. artemidis* is negative for acid from lactose. *S. noxia* is negative for all of the previously mentioned traits of *Selenomonas* species, but growth is abundant in a peptone-yeast extract medium containing glucose, mannose, sorbitol, or sorbose.

Phylogeny

The GC content of the DNA from *Selenomonas* species ranges from 48 to 58 mol% (see Table 1). *S. acidaminophila* has the lowest value, whereas *S. ruminantium* strains vary from 49 to almost 54 mol%. The oral species of *Selenomonas* have only a narrow range of 53 to 58 mol%, but it is clear from the results of DNA-DNA hybridization (Table 3) that these strains are, in fact, separate species.

The phylogeny of species of *Selenomonas* and related bacteria has been determined by using 16S rRNA sequence analysis (Dewhirst et al., 1989). Complete 16S rRNA sequences of these bacteria were compared with the rRNA sequences of over 250 other bacterial species (Paster and Dewhirst, unpublished observations). From these data, a phylogenetic tree was constructed (Fig. 1). The microorganisms tested fall into two major groups—*Selenomonas* and related bacteria occupy one branch, and *Veillonella* and related bacteria are on the other. The *Selenomonas* group is phylogenetically coherent with interspecies homology levels of 90 to 99%. *Selenomonas dianae*, *S. infelix*, *S. flueggei*, *S. noxia*, *S. artemidis*, and *Centipeda periodontii* form a very tight cluster with a homology range of 96 to 99%. *Selenomonas sputigena* and *S. ruminantium* have an average sequence homology of 94% with members of this cluster. Aside from its unusual flagellation, *C. periodontii* is phenotypically similar to other members of the genus *Selenomonas*.

Pectinatus cerevisiiphilus, a Gram-negative, anaerobic, motile rod originally isolated from spoiled beer, is related to the *Selenomonas* group, but with an average homology value of only 91% (for further information see Chapter 91). *Sporomusa paucivorans* is an anaerobic, Gram-negative, sporeforming rod. This organism is related to the selenomonads at an average homology level of 90%.

In the other major branch of this tree, *Veillonella dispar* and *V. parvula*—aerobic, Gram-negative cocci isolated from humans—are very closely related to each other with 99% sequence homology. *Megasphaera elsdenii* (see *Pectinatus*, *Megashaera* and *Zymophilus* in this Volume), an anaerobic, large, Gram-negative coccus isolated from humans and the ovine rumen, is related to *Veillonella* at a level of 92% homology. Species within these two bacterial genera are related to members of the *Selenomonas* branch with an average homology of 88%. The close relationship between motile, curved rods and nonmotile cocci may seem unusual, but there are phenotypic traits that unify this diverse group. One of the more convincing characteristics is that species of *Selenomonas*, *Sporomusa*, *Veillonella*, and *Megasphaera* all possess the one of the diamines, either cadaverine or putrescine, which are covalently bound to their peptidoglycan (Stackebrandt et al., 1985).

It has been previously shown that the genera *Selenomonas* and *Veillonella* share a branch with the Gram-positive bacteria (Stackebrandt et al., 1985). Members of these two groups both have an average 16S rRNA sequence homology of 85% with the Gram-positive bacteria, as represented by species of *Clostridium*, *Bacillus*, and *Enterococcus*, but only 79% sequence homology with Gram-negative bacteria such as *E. coli* and related bacteria (Fig. 2). Other bacterial groups, such as those containing the spirochetes, bacteroides, and radiation-resistant bacteria, branch deeper in the tree (unpublished observations) with even lower sequence homologies (e.g., <75%) with the selenomanads. From these data, it is evident that the selenomonads should be removed from the *Bacteroidaceae,* their current taxonomic placement in *Bergey's Manual of Systematic Bacteriology*. In general, a sequence homology of less than 80% indicates that the bacteria compared are in different major taxonomic divisions.

Table 3. Percentage of homology from DNA-DNA hybridizations of oral *Selenomonas* species.

Species	S. artemidis	S. noxia	S. flueggei	S. infelix	S. sputigena	S. dianae
S. artemidis	100					
S. noxia	24	100				
S. flueggei	13	22	100			
S. infelix	15	20	18	100		
S. sputigena	4–8	13	5	6	100	
S. dianae	14	28	15	30	4–8	100

Data from Moore et al. (1987).
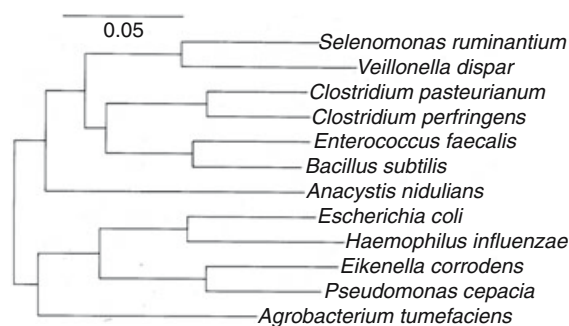
Fig. 2. Phylogenetic tree for the selenomonads, related bacteria, and distantly related bacteria. (Sequences for *C. perfringens* and *E. faecalis* courtesy of C. R. Woese.)

Now that 16S rRNA sequences are known (see below), it will be possible to develop short DNA probes targeted for signature regions of 16S rRNA. These probes can be used to identify new strains or species isolated from environmental samples. Ultimately, these probes will allow for the rapid identification of selenomonads directly from samples without in vitro cultivation. Family-specific and species-specific DNA probes have been already used to identify RNA isolated from selenomonads (Dewhirst et al., 1989).

Physiological Traits

Many biochemical and physiological studies have been done with *S. ruminantium*, but not with the oral species of *Selenomonas*. Early studies on isolation of ruminal bacteria did not indicate that *S. ruminantium* produced hydrogen gas as a fermentation product. However, when this organism was grown in the presence of a hydrogen-utilizing bacterium, namely a methanogen, (Scheifinger et al., 1975b). As a consequence, considerably less lactate and propionate were produced from glucose fermentation. *S. ruminantium* also changes the array of fermentation acids made as function of growth rate, with acetate and propionate dominating at low growth rates and lactate at high growth rates (Hobson, 1965). These shifts in fermentation products are correlated with less ATP formation and can be largely explained by the effects of pyruvate on the activity of the intracellular lactic acid dehydrogenase (Scheifinger et al., 1975a; Wallace, 1978). At higher growth rates, intracellular pyruvate pools probably increase and this compound causes a homotropic activation of lactate acid dehydrogenase, resulting in higher rates of lactate formation. The other major fermentation acid, propionate, has been shown to be formed via the succinate pathway (Paytner and Elsden, 1970). Propionate formation most likely involves generation of ATP via formation of a proton-motive force, since *S. ruminantium* was one of the first anaerobes to be shown to possess cytochromes, mainly of the cytochrome-*b* type (DeVries et al., 1974).

Ammonia and urea play central roles in ruminal nitrogen metabolism. *S. ruminantium* is one of the major ureolytic bacteria in the rumen (Wozny et al., 1977). Studies on ammonia assimilation and glutamate formation in *S. ruminantium* indicate this organism possesses both the glutamate dehydrogenase and glutamine synthetase pathways for ammonia assimilation (Smith et al., 1980). In fact, this study was the first to show presence of a nonadenylylation control mechanism for glutamine synthetase in a Gram-negative organism. However, it was shown later that this enzyme and urease were coordinately controlled (Smith et al., 1981), as in other bacteria.

One of the first detailed studies on survival of anaerobic bacteria under nongrowing conditions was with *S. ruminantium*. Washed cell suspensions obtained from glucose-limited chemostats were found to lose viability very rapidly, with about a 50% loss in 2.5 hours (Mink and Hespell, 1981). The viability losses could not be attributed to cell lysis but correlated with rapid declines in cellular DNA, RNA, and protein. Similar effects were found with cells grown under nitrogen-limited conditions but better survival times were observed that correlated with growth rates (Mink et al., 1982). During starvation, the cells produced trace amounts of acetate as the only fermentation product. In addition, it was shown that the cellular levels of urease, glutamine synthetase, and glutamate dehydrogenase remained relatively stable despite loss of cell viability.

Applications

At present, no biotechnological applications have been made with *Selenomonas* species. However, a recent paper on the kinetics of glucose fermentation by *S. ruminantium* suggests that this organism might have commercial application for the production of lactic acid (Shimizu et al., 1989). A new species of the genus *Selenomonas* has been described by Schleifer et al. 1990. *Selenomonas lacticifex* was isolated from pitching yeast and can ferment glucose to lactic acid as major product.

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CHAPTER 1.3.4

The Genus *Sporomusa*

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Introduction

The genus *Sporomusa* was created in 1984 (Möller et al., 1984) to accommodate a number of strains of anaerobic, homoacetogenic bacteria distinguished by having a Gram-negative cell wall and an ability to form endospores-two properties whose coincidence is uncommon in the microbial world. The genus name means "spore-bearing banana," and reflects the slightly curved, rod shape of the cells. Two species, *S. sphaeroides* and *S. ovata*, were distinguished from each other on the bases of endospore shape, substrate utilization pattern, and G+C content of their genomic DNA. Subsequently, five additional species were revealed, and the phylogenetic position of *Sporomusa* was clarified. This chapter will update our understanding of *Sporomusa*, including several of their more notable properties: their common occurrence in anoxic (and even seemingly well-aerated) environments, their ability to grow by decarboxylation of organic acids, and their possession of unusual corrinoids.

Phylogeny

The first insight into the phylogeny of *Sporomusa* came from analysis of 16S rRNA oligonucleotide catalogues (Stackebrandt et al., 1985). Results revealed that *S. sphaeroides* and *S. ovata* bore a distinct, but remote, relationship to the Gram-negative bacteria, *Selenomonas* and *Megasphaera*, and to Gram-positive bacteria of the "*Clostridium* subdivision." This was borne out in subsequent analyses of additional species, and in a comprehensive reexamination of the phylogeny of the genus *Clostridium* and close relatives. Collins et al. (1994) assigned *Sporomusa* to "Cluster IX"—a deeply branching assemblage of bacteria in the "Clostridium phylum"-along with a heterogeneous collection of other spore-forming and nonspore-forming organisms, many of which were Gram negative. At that time, Cluster IX included *Acidaminococcus*, *Clostridium quercicolum* (now *Dendrosporobacter quercicolus*; Strömpl et al., 2000), *Megasphaera*, *Pectinatus*, *Phascolarctobacte-*

rium, *Quinella*, *Selenomonas* and *Zymophilus paucivorans*. It has since become clear that a number of other isolates obtained over the past ten years can be added to this cluster, including *Acetonema* (Kane and Breznak, 1991), *Anaeromusa* (Baena et al., 1999), *Anaeroarcus*, *Anaerosinus* and *Anaerovibrio* (Strömpl et al., 1999), *Centipeda periodontii* (Sawada et al., 1999), *Dialister* (Willems and Collins, 1995b), *Mitsuokella multacida* (Willems and Collins, 1995a), *Propionispora* (Biebl et al., 2000), *Succiniclasticum* (Van Gylswyk, 1995), *Succinispira* (Janssen and O'Farrell, 1999), *Schwartzia* (Van Gylswyk et al., 1997), and *Veillonella* (Willems and Collins, 1995b). This phylogenetic group is sometimes referred to as the "*Sporomusa* branch" (Willems and Collins, 1995b), the "*Sporomusa-Pectinatus-Selenomonas* group" (Biebl et al., 2000), or by similar phrases.

Taxonomy

In the forthcoming volume 2 of the second edition of *Bergey's Manual of Systematic Bacteriology*, *Sporomusa* will likely be regarded as a genus within Family VII (Acidaminococcaceae) of Order I (Clostridiales) of class I ("Clostridia") of Phylum BXIII (Firmicutes) of the Domain Bacteria (Garrity and Holt, 2000). At this writing, the genus *Sporomusa* contains seven named species. Phenotypic properties useful in distinguishing between them are given in Table 1.

Habitat

Sporomusa has been isolated from anoxic sediments of freshwater rivers, lakes, creeks and ditches (Möller et al., 1984; Hermann et al., 1987; Dehning et al., 1989; Sass et al., 1998), and anoxic soil from rice paddy microcosms (Rosencrantz et al., 1999); from soils and silage (Möller et al., 1984; Kuhner et al., 1997); from sugar beet factory and distillery wastewater (Möller et al., 1984; Ollivier et al., 1985); from horse and cattle dung (Möller et al., 1984); and from the gut of

Table 1. Properties useful in differentiating species of the genus *Sporomusa*.

Property	*S. sphaeroides*	*S. ovata*	*S. acidovorans*	*S. paucivorans*	*S. termitida*	*S. malonica*	*S. silvacetica*
Cell size (μm)	0.5–0.8 × 2–4	0.7–0.9 × 3–5	0.7 × 5	0.4–0.7 × 2–3	0.5–0.8 × 2–8	0.7 × 2.6–4.8	0.7 × 3.5
Endospores	+	+	+	–	+	+	+
Motility	+	+	+	+	+	+	+
Catalase	+	(–)	ND	–	+	–	–
Utilization of:							
H$_2$+CO$_2$	+	+	+	+	+	+	+
Methanol	+	+	+	+	+	+	+
Formate	+	+	+	+	+	+	+
Betaine	+	+	ND	+	+	+	+
Fructose	–	+	+	–	–	+	+
Mannitol	–	–	ND	–	+	ND	–
Glycerol	+	–	+	+	–	–	+
Propanol	+	+	ND	+	–	+	ND
Citrate	–	–	–	–	+	+	–
Fumarate	–	–	+	–	–	+	+
Malate	ND	ND	+	–	–	+	ND
Succinate	–	–	+	–	+	+	–
G+C content (mol%)	46.7–47.4	41.3–43.3	42	47.1	48.6	44.1	42.7

Symbols: +, positive; –, negative; (–), negative or very weak reaction; and ND, not determined.

wood-eating termites (Breznak and Switzer, 1986; Breznak et al., 1988). Inocula for the isolations cited above were obtained from many parts of the world. No reports exist of *Sporomusa* strains isolated from thermal habitats or from brackish or marine habitats, although there is no reason to think that such strains should not exist. Indeed, *S. malonica* was found to grow in a "brackish water" medium containing 10 g of NaCl and 1.5 g of $MgCl_2 \cdot 6 H_2O$ per liter (Dehning et al., 1989).

Isolation

Enrichment Cultures

At present there is no enrichment procedure that is entirely specific for *Sporomusa*. However, selectivity can in principle be enhanced by exploiting the resistance to heat that is typical of bacterial endospores, followed by inoculating the heat-treated source material into an anoxic medium that is low in sulfate (to discourage growth of sulfate-reducing bacteria) and that contains a substrate reasonably selective for *Sporomusa*. Heat treatment is done by pasteurization at 80°C for 10–15 minutes. A variety of substrates can be used as energy sources by one or more species of *Sporomusa*, including molecular hydrogen (H_2) + carbon dioxide (CO_2), various sugars (e.g., fructose), organic acids (e.g., lactate), glycols (e.g., ethylene glycol, 2,3-butanediol), alcohols (e.g., methanol, ethanol), and *N*-methyl (e.g., betaine) and *O*-methyl (e.g., methoxylated aromatic) compounds. However, in liquid enrichment cultures, *Sporomusa* might be accompanied by clostridia or other endospore-forming bacteria (e.g., *Acetonema*; Kane and Breznak, 1991), that also use some of these same compounds. Substrates that support good growth of all species of *Sporomusa* are $H_2 + CO_2$ ($H_2/CO_2 \geq 2/1$, v/v) and methanol + CO_2 (molar ratio of methanol/$CO_2 \geq 2/1$), which are used by all species of the genus. When methanol is used, the concentration in the medium should be ≤ 5 mM (to avoid toxicity) and replenished as necessary. Whether these or other selective substrates are employed, CO_2 should constitute 20% or more of the gas phase, wherein it can serve as: 1) a major C source for acetogenesis (e.g., with H_2 or ethanol as the oxidizable substrate); 2) a ready source of the carboxyl group of acetate (e.g., in combination with substrates such as methanol, betaine or methoxylated aromatics, which provide preformed methyl groups); or 3) a readily available substrate for carboxylation reactions involved in biosynthesis. Carbon dioxide in the gas phase should be balanced by an appropriate amount of HCO_3^- in the liquid phase

to buffer the medium at pH 7 (Breznak and Costilow, 1994b), which is at or near the pH optimum of all strains tested. An incubation temperature of 30–35°C will permit growth of all known species of *Sporomusa*. Butyl rubber-stoppered tubes or bottles for isolation and enrichment cultures are those used for cultivation of methanogens and other anaerobes and are available commercially (Miller and Wolin, 1974; Balch and Wolfe, 1976; Hermann et al., 1986).

The one-carbon compounds formate and carbon monoxide (CO) are also used by some species of *Sporomusa*, but growth with formate is sometimes poor, whereas CO can be toxic. Consequently, growth and/or acetate production above that in control (no substrate) enrichments may be difficult to detect with these substrates. Targeted enrichment and isolation of *S. acidovorans*, *S. malonica* and *S. termitida* is, in principle, possible by using sodium succinate as an energy source. These species can grow by decarboxylation of succinate to propionate $+CO_2$. *Spormusa malonica* and *S. termitida* also will grow by decarboxylation of malonate to acetate $+CO_2$. However, the energy yield from such decarboxylations is low, so the growth rate and yield of cells per mole of substrate will also be low. The use of *N*-methyl compounds (e.g., betaine) and aryl *O*-methyl compounds (e.g., 3,4,5-trimethoxybenzoate) as enrichment substrates may also provide some selectivity. They are a ready source of the methyl-group of acetate by demethylation and, by oxidation of some of the released methyl groups, of electrons for CO_2 reduction to the carboxyl group of acetate. Betaine was used by pure cultures of all species tested, i.e., *S. sphaeroides* and *S. ovata* (Möller et al., 1984; Kamlage et al., 1993b), *S. paucivorans* (Hermann et al., 1987), *S. termitida* (Breznak et al., 1988) and *S. silvacetica* (Kuhner et al., 1997). Likewise, *O*-methylated aryl substrates were used by *S. ovata* (Stupperich and Konle, 1993), *S. silvacetica* (Kuhner et al., 1997) and *S. termitida* (Breznak et al., 1988).

For enrichment of *S. paucivorans* (the only species of *Sporomusa* that does not appear to form endospores; Hermann et al., 1987), or for ecological studies where all viable *Sporomusa* cells are to be enumerated by using most probable number (MPN)-type dilution series, heat treatment of the inoculum (e.g., pasteurization) should be avoided. However, it is worth noting that with the exception of *S. sphaeroides* and *S. ovata*, most other species of *Sporomusa* were isolated from enrichment cultures established with unpasteurized inocula and $H_2 + CO_2$ or methanol + CO_2 as substrates (*S. malonica* was obtained from a glutarate-degrading consortium and then isolated in agar medium with crotonate as a substrate; Dehning et al., 1989). Following

inoculation, liquid cultures are monitored for growth of putative *Sporomusa* by noting the development of turbidity; by microscopic examination for cells with a morphology that is typical of *Sporomusa* (Fig. 1); and by assay for acetate production (or propionate production if succinate is the substrate). The latter assays can be done by gas chromatography or by high performance liquid chromatography of acidified samples of culture supernatant fluid (see Breznak and Switzer, 1986). If H_2+CO_2 is used as substrate, consumption of this gas mixture is also presumptive evidence of homoacetogenesis and results in the development of negative pressure in the headspace of culture tubes. This can be examined by inserting an H_2/CO_2-filled glass syringe (5 cc or larger capacity, whose barrel has been lubricated with a small amount of light silicone oil) equipped with a 25-gauge needle through the rubber stopper of the culture vessel and by noting the extent to which the plunger moves downward. This simultaneously replenishes the gas used.

A potential confounding factor in enrichment/enumeration of *Sporomusa* is, of course, overgrowth by other homoacetogens, many of which use the same substrates and may be present in the inoculum in greater numbers than *Sporomusa*, or simply grow faster under the conditions employed. With H_2+CO_2, methanol $+CO_2$, or *N*-methyl compounds as substrates, an additional confounding factor is the possible overgrowth of *Sporomusa* by methanogens. To minimize this latter possibility, a specific inhibitor of methanogens, e.g., 2-bromoethanesulfonate (BES) at 1–50 mM (Oremland, 1988), may be included in the medium. However, as yet no systematic studies have been made of the efficacy of BES in media for enrichment of *Sporomusa* on methanogenic substrates. Interestingly, with some substrates, the purposeful addition of methanogens to enrichments actually appears to enhance the recovery of *Sporomusa*. For example, Rosenkrantz et al. (1999) used ethylene glycol, 2,3-butanediol, and 3,4,5-trimethoxybenzoate as substrates in liquid media for MPN-type enumeration of homoacetogens from anoxic rice paddy soils; and from the highest dilution tubes showing growth, strains of *Sporomusa* were isolated. However, the addition of *Methanospirillum hungatei* to analogous tubes with lactate or ethanol as substrate yielded counts of homoacetogens that were several orders of magnitude higher, and from the highest dilution tubes, strains of *Sporomusa* were isolated that were able to use the former three substrates when in pure culture. Why these strains did not initiate growth in higher dilution tubes containing those three substrates in the first place (without exogenously added *M. hungatei*) is not known; neither is the basis for the growth-promoting effect of *M. hungatei* on *Sporomusa*.

Isolation

Isolation of *Sporomusa* is done by streaking the surface of anoxic agar media in "bottle plates" (Hermann et al., 1986) or by diluting samples through a series of "roll tubes" or "shake tubes," which are, in effect, pour plates in a tube. For the former, tubes containing inoculated and mixed molten agar medium are held horizontally under cold water and rolled as the agar solidifies, resulting in a shell of agar medium on the inside surface of the tube. For the latter, the agar is allowed to solidify while the tube is held in a vertical position. When solidified, shake tubes are incubated vertically, but upside (i.e., mouth-side) down. This is done so that the water of syneresis exuded from the agar as it solidifies does not accumulate on the agar surface, through which sterile Pasteur pipettes (with ends drawn out to a tapered capillary) are later inserted to pick isolated subsurface colonies. A detailed description of the roll tube technique is given by Hungate (1969), who also discusses the theoretical and practical aspects of preparation of anoxic medium and methods for the cultivation and manipulation of strict anaerobes in general. Isolation media are then examined for colonies of a size (0.5–4 mm) and morphology (round with an entire margin; white to dark brown) similar to that of known species of *Sporomusa*. Suspected *Sporomusa* colonies are picked, suspended in a small amount of sterile anoxic medium, and
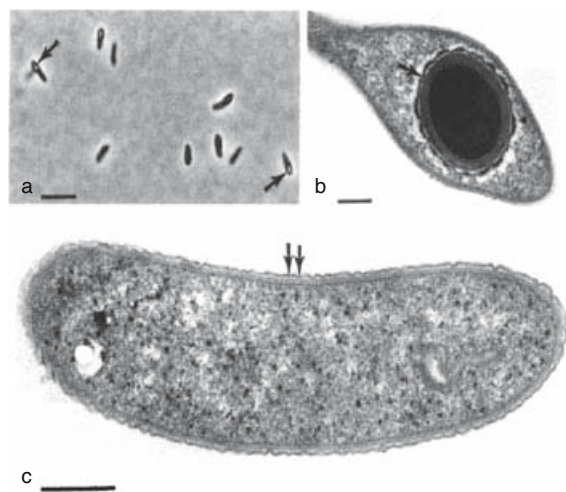
Fig. 1. Morphology of *Sporomusa termitida* strain JSN-2. (a) Phase-contrast micrograph. Bar = 10 μm. (b), (c) Transmission electron micrographs of thin sections. Bars = 0.25 μm. Note endospores (a, b, single arrows) and the outer membrane of the cell wall (c, double arrows). (From Breznak et al., 1988.)

examined by phase contrast microscopy for typical *Sporomusa* cells (Fig. 1a). Smears also can be heat-fixed and Gram-stained for bright field microscopic examination. Presumptive *Sporomusa* colonies are then used as inocula to repeat the isolation procedure until the purity of the cultures is confirmed and the isolates can be completely characterized. If agar media containing H_2+CO_2 as substrate are held in rubber-stoppered tubes or bottles, efforts may be focused on those colonies present in vessels that develop negative pressure in the headspace. Inclusion of a pH indicator in agar media (e.g., 0.01% bromcresol green) may aid in recognizing acid-producing colonies of *Sporomusa* (Braun et al., 1979).

Media

Various culture media have been used for *Sporomusa*: all are more or less similar in composition. As yet no detailed nutritional studies have been done to define the vitamin and/or trace element requirement of individual species. The composition of a basal medium used to enrich for, isolate, and cultivate *S. sphaeroides* and *S. ovata* follows as an example. It would probably, with little or no modification, support the growth of all known species.

Basal Medium for *S. sphaeroides* and *S. ovata* (Möller et al., 1984)

K_2HPO_4	0.348 g
KH_2PO_4	0.227 g
NH_4Cl	0.500 g
$MgSO_4 \cdot 7H_2O$	0.500 g
$CaCl_2 \cdot 2H_2O$	0.025 g
NaCl	2.250 g
$FeSO_4 \cdot 7H_2O$	2 mg
$NaHSeO_3$	15 µg
SL-10 trace element solution (below)	3 ml
Resazurin	1 mg
Distilled water	905 ml

The mixture is deoxygenated by boiling for 5 min, then it is cooled in an ice bath under a stream of nitrogen (N_2)/CO_2 (80/20, v/v) until room temperature is reached. At this point are added 80 ml of $NaHCO_3$ solution (4 g of $NaHCO_3$ in 80 ml of distilled water; gassed with N_2/CO_2 for 20 min) and 2 ml of a 10X stock vitamin solution (below). The medium is then dispensed in tubes or bottles in 9.9 ml amounts (or multiples thereof), sealed under N_2/CO_2 with rubber (preferably butyl rubber) stoppers, and sterilized by autoclaving at 121°C for 20 min. Before use, each 9.9 ml of basal medium is reduced by injecting through the stopper 0.1 ml of Na_2S solution (30 g of $Na_2S \cdot 9H_2O$ per liter distilled water; sterilized by autoclaving under N_2; final concentration in the medium is 1.25 mM) or cysteine solution (30 g of L-cysteine · HCl monohydrate per liter of distilled water; sterilized by autoclaving under N_2; final concentration in the medium is 1.71 mM). This should result in the decolorization of pink resorufin (formed from resazurin upon heating) indicating that the redox potential of the medium is below –51 mV.

SL-10 Trace Mineral Solution (Widdel et al., 1983)

HCl (25% w/v)	10 ml
$FeCl_2 \cdot 4H_2O$	1.5 g
$CoCl_2 \cdot 6H_2O$	190 mg
$MnCl_2 \cdot 4H_2O$	100 mg
$ZnCl_2$	70 mg
H_3BO_3	6 mg
$Na_2MoO_4 \cdot 2H_2O$	36 mg
$NiCl_2 \cdot 6H_2O$	24 mg
$CuCl_2 \cdot 2H_2O$	2 mg
Distilled water	990 ml

The $FeCl_3$ is dissolved in the HCl first, then distilled water is added and the other salts are sequentially dissolved.

10X Stock Vitamin Solution (Wolin et al., 1964)

Biotin	20 mg
Folic acid	20 mg
Pyridoxine HCl	100 mg
Thiamine HCl	50 mg
Riboflavin	50 mg
Nicotinic acid	50 mg
Calcium pantothenate	50 mg
Para-aminobenzoic acid	50 mg
Thioctic acid	10 mg
Vitamin B_{12}	0.1 mg
Distilled water	1,000 ml

When H_2/CO_2 is used as substrate, the medium can be dispensed into culture tubes or bottles under H_2/CO_2 instead of N_2/CO_2 before sterilization. When other substrates are used as energy sources, they are prepared separately as concentrated anoxic stock solutions, sterilized by autoclaving or filtration, and added to the basal medium at a final concentration of 5–50 mM. In this case, a slight adjustment may be made in the initial volume of distilled water used to prepare the basal medium so as to accommodate the volume of stock substrate added later. For solid medium, agar is included at a final concentration of 10–20 g per liter (the lower concentrations preferred for shake tubes; the higher concentrations preferred for roll tubes or bottle plates).

Additional Comments on Media Preparation, Isolation and Cultivation

Degassing of basal medium and other aqueous solutions used for medium preparation also can be achieved by using a water faucet vacuum aspirator for 3–5 minutes while the solution is stirred with a magnetic stirring bar or is swirled by hand. The degassed solution is then held under an O_2-free gas for further manipulations prior to, and following, sterilization. This method is faster than boiling, it eliminates the cooling down time, and it is safer. However, liquids must be held in a vacuum flask, or thick-walled serum bottle or tube during degassing to prevent implosion of the vessel.

Most species of *Sporomusa* require, or are greatly stimulated by, yeast extract and a source

of amino acids. Hence, inclusion in the medium of yeast extract and trypticase, casitone, or casamino acids, at a concentration of 0.5–1 g per liter, may well increase the chances for successful enrichment and isolation of pure cultures.

Although Na$_2$S or cysteine (at 1–2 mM final concentration) are the reducing agents most often used for cultivation of *Sporomusa*, *S. termitida* could not be enriched in media containing these compounds or various alternatives (e.g., titanium citrate, dithiothreitol, amorphous ferrous sulfide, thioglycollate or ascorbic acid). Successful enrichment and isolation of this species was only achieved by using palladium chloride (PdCl$_2$) powder (330 mg per liter) as a catalyst for reduction of the medium by H$_2$, which was a component of the H$_2$+CO$_2$ substrate mixture. After pure cultures were obtained, they could be adapted to grow with dithiothreitol (1 mM final concentration) or other reducing agents (Breznak et al., 1988). Thus, unsuccessful enrichment or isolation of *Sporomusa* might be due to the reducing agent used. It is noteworthy that growth of *S. ovata* is inhibited at sulfide concentrations of 2 mM or greater (Heijthuijsen and Hansen, 1986).

Identification

Recognition of an isolate as a strain of *Sporomusa* is based on: 1) the size (0.4–0.9 × 2–8 mm) and usually curved shape of cells; 2) the formation of endospores by most species; 3) the true Gram-negative character of the cell wall layer; 4) the production of acetic acid as the sole or major product of the fermentation of most substrates, including one-carbon compounds such as H$_2$+CO$_2$, and methanol (+CO$_2$); and 5) their phylogenetic position based on 16S rRNA sequence. None of the named species is known to respire anaerobically with NO$_3^-$ or SO$_4^{2-}$. However, five as-yet-unnamed strains of sulfate-reducing bacteria affiliated with the *Sporomusa* cluster were isolated from an oligotrophic freshwater lake in Germany, and a partial (626-bp) sequence of their 16S rDNA gene revealed *S. termitida* to be their closest relative (95% similarity; Sass et al., 1998). *Sporomusa paucivorans* can reduce sulfide (S0) and cystine, but it does not carry out energy-yielding dissimilatory reduction of these compounds (Hermann et al., 1987). *Sporomusa ovata* is able to dechlorinate tetrachloroethylene (PCE) to trichloroethylene during growth on methanol +CO$_2$, but this does not affect the growth of cells, suggesting that energy cannot be conserved by halorespiration (Terzenbach and Blaut, 1994).

The morphology of *S. termitida* is shown in Fig. 1 as an example. Cells of *Sporomusa* possess a distinct outer membrane, however, it is not yet known whether such membranes contain lipopolysaccharide. Putrescine, cadaverine and spermidine were the major cellular polyamines in a number of *Sporomusa* species; and it appeared that some of these polyamines were covalently linked to peptidoglycan (Hamana, 1999). Vegetative cells are motile by up to 15 flagella inserted laterally. This arrangement imparts a characteristic tumbling type of motility to cells. In *S. sphaeroides*, flagellar rotation was shown to be driven by an electrochemical proton potential (Müller and Bowien, 1995). Membrane-bound cytochromes of the *b*-type and *c*-type (Möller et al., 1984; Breznak et al., 1988; Dehning et al., 1989; Kamlage et al., 1993b; Kuhner et al., 1997) and a respiratory lipoquinone (lipid F; Stackebrandt et al., 1985) have been found in some species. The presence of lipid F appears to be a common characteristic of members of the *Sporomusa-Pectinatus-Selenomonas* phyletic group (Strömpl et al., 2000). In addition, *S. ovata* has been found to possess two unique corrinoids, *p*-cresolyl cobamide (Stupperich et al., 1988) and phenolyl cobamide (Stupperich et al., 1989), which are present in these cells in considerable amounts. These corrinoids differ from all others isolated so far from nature in that their nucleotides consist of phenols rather than a nucleotide base. They appear to be cofactors involved in methyl transfer reactions accompanying the metabolism of methanol and 3,4-dimethoxybenzoate (Stupperich and Konle, 1993). Preliminary analysis of *S. malonica* suggests that it too may contain one or more of these types of corrinoids (Dehning et al., 1989). Endospores of *Sporomusa* are either spherical or oval, terminal or subterminal, and may distend the sporangium (Figs. 1a and c). They are heat-resistant (80°C for 10 min) and contain dipicolinic acid (Breznak et al., 1988; Kuhner et al., 1997).

An H$_2$/CO$_2$-utilizing acetogen that could be mistaken for *Sporomusa* is *Acetonema*, the first and currently only species of which (*A. longum*) was isolated from gut contents of the dry-wood termite, *Pterotermes occidentis* (Kane and Breznak, 1991). *Acetonema longum* is similar to *Sporomusa* in possessing endospores, as well as a Gram-negative-type cell wall. However, *A. longum* is distinct from known species of *Sporomusa* morphologically in having a high cell length-to-width ratio (cells measure 6–60 mm long × 0.3–0.4 mm wide). Moreover, *A. longum* forms abundant amounts of butyrate during glucose fermentation (ca. 1 mol butyrate per mol glucose fermented). *Acetonema longum* also groups within the *Sporomusa-Pectinatus-Selenomonas* group by 16S rRNA sequence analysis, but it sufficiently different from *Sporomusa* phy-

logenetically to warrant recognition as a distinct genus (Kane and Breznak, 1991; Biebl et al., 2000). Other Gram-negative sporeforming anaerobes that resemble *Sporomusa* morphologically are *Dendrosporobacter quercicolus* (Stankewich et al., 1971; Strömpl et al., 2000), *Propionispora vibrioides* (Biebl et al., 2000), and *Sporohalobacter* (Oren et al., 1987). The former two bacteria are also in the *Sporomusa-Pectinatus-Selenomonas* phylogenetic group, but neither uses H_2+CO_2 or one-carbon compounds as energy sources, nor are they homoacetogens. Each produces propionate and acetate from the fermentation of fructose and polyols. Members of *Sporohalobacter* are halophilic anaerobes that produce a variety of products from glucose fermentation and have a relatively low G+C content in their genomic DNA (29.6–31.5%). Analysis of 16S rRNA oligonucleotide catalogues indicated that they were not closely related to *Sporomusa*.

Preservation

The ability of most species of *Sporomusa* to form endospores suggests that sporulated cultures may be stored without special additional treatment for extended periods. Viable subcultures of *S. termitida* have been obtained from broth cultures kept at room temperature for over a year, cultures in which only endospores and ghostlike remnants of vegetative cells were seen (J. A. Breznak, unpublished observation). Hermann et al. (1987) maintained stock cultures of the nonsporing *S. paucivorans* by freezing cells at −80°C in growth medium containing 20% glycerol. Viable *S. termitida* also has been retrieved from cell suspensions frozen at −60°C in fresh medium containing 4% (v/v) dimethyl sulfoxide (J. A. Breznak, unpublished observation).

Physiology

The pathway of acetate formation from one-carbon compounds in *Sporomusa* appears to be the "acetyl CoA pathway," also referred to as the "Wood-Ljungdahl pathway" (Drake, 1994). Virtually all of the enzyme activities associated with this pathway have been demonstrated in *S. sphaeroides*, including CO dehydrogenase, hydrogenase, formate dehydrogenase, formyl-tetrahydrofolate (THF) synthetase, methenyl-THF cyclohydrolase, methylene-THF dehydrogenase, methylene-THF reductase, and an activity capable of ^{14}C-acetate synthesis from $^{14}CH_3$-THF, implying the presence of methyltransferase (Kamlage and Blaut, 1993a; Kamlage et al., 1993b). A methyl transferase activity was

demonstrated in *S. ovata* that transferred the methyl group from methyl-THF to cob(I)alamin in vitro; and two serologically different, corrinoid-dependent *O*-methyl transferases were involved in transferring the methyl group to THF during metabolism of methanol and 3,4-dimethoxybenzoate (Stupperich et al., 1992; Stupperich and Konle, 1993). The methyltransferase involved in methanol metabolism is a 40-kDa protein with firmly bound *p*-cresolyl cobamide. Both CO dehydrogenase and hydrogenase activities were also demonstrated in cell extracts of *S. silvacetica* (Kuhner et al., 1997), and formyl-THF synthetase activity was present in *S. termitida* at a specific activity of 0.084 ± 0.040 U/mg protein (n = 3) when cells were grown on H_2+CO_2 (J. A. Breznak, unpublished observation). Carbonic anhydrase activities varied widely between *S. silvacetica* (0.8 U/mg protein) and *S. termitida* (below the detection limit of the assay), so the importance of this activity to acetogenesis and energy conservation in *Sporomusa* is not entirely clear (Braus-Stromeyer et al., 1997).

The total synthesis of acetate from H_2+CO_2 is an exergonic reaction with a large enough change in free energy to support the growth of all species of *Sporomusa* (equation 1).

$$4H_2 + 2HCO_3^- + H^+ \rightarrow acetate^- + 4H_2O$$
$$(\Delta G^{0\prime} = -104.6 \text{ kJ}) \tag{1}$$
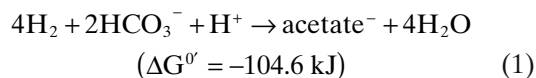
In the Wood/Ljungdahl pathway, one ATP is consumed in the formyl-THF synthetase reaction during reduction of one molecule of CO_2 to the methyl group; and one ATP is formed by substrate-level phosphorylation during conversion of acetyl phosphate to acetate via acetate kinase. Hence, there remains a need for net energy conservation during autotrophic growth, and this is most likely achieved through an ion-translocating ATPase driven in the direction of ATP synthesis by an electrochemical transmembrane ion gradient formed during the reduction of methylene-THF to methyl-THF by methylene-THF reductase (Drake, 1994). In *S. sphaeroides*, cytochromes appear to function as a physiological electron donor for methylene-THF reductase during autotrophic growth, and as an electron acceptor for methyl group oxidation during growth on methanol or other methyl-containing compounds (Kamlage and Blaut, 1993a).

Sporomusa sphaeroides possesses two types of hydrogenase: a soluble activity that mediates electron transfer from H_2 to $NADP^+$, and a membrane-bound, [NiFe] uptake hydrogenase (Dobrindt and Blaut, 1996). The former provides NADPH for the formate dehydrogenase and methylene-THF dehydrogenase reactions. The immediate physiological electron acceptor for

the latter remains to be identified, but the role of this hydrogenase appears to be the donation of electrons to the membrane-bound electron transport chain, inasmuch as washed cell membranes from *S. sphaeroides* mediate an H_2-dependent reduction of a *b*-type cytochrome(s) in the membrane. One working model assumes that generation of a transmembrane electrochemical proton gradient during autotrophic growth results from proton pumping that accompanies electron transfer from H_2 to methylene-THF via cytochromes, or by the release of scalar protons at the periplasmic side of the membrane (Dobrindt and Blaut, 1996).

All species of *Sporomusa* ferment methanol to acetate, but exogenous CO_2/HCO_3^- is required as an electron acceptor for good growth and fermentation of this substrate, as depicted in equation 2.

$$4CH_3OH + 2HCO_3^- \rightarrow 3 \text{ acetate}^- + H^+ + 4H_2O$$
$$(\Delta G^{0'} = -219.8 \text{ kJ}) \qquad (2)$$
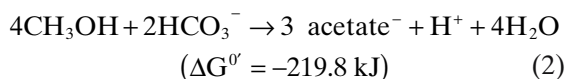
In theory, fermentation of methanol should be possible without exogenous CO_2/HCO_3^- if excess reducing equivalents are evolved as H_2 (equation 3).

$$4CH_3OH \rightarrow 2 \text{ acetate}^- + 4H_2 + 2H^+$$
$$(\Delta G^{0'} = -115.2 \text{ kJ}) \qquad (3)$$
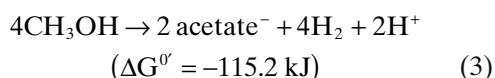
However, as the carboxyl group of acetate arises from the oxidation of methanol via THF intermediates, the reduction of protons to H_2 ($E^{0'} = -414 \text{ mV}$) with electrons derived from the oxidation of methyl-THF to methylene-THF ($E^{0'} = -200 \text{ mV}$; Wohlfarth and Diekert, 1991) may require too much ATP to make this option energetically feasible for growth. This concept is discussed in more detail by Heijthuijsen and Hansen (1986).

A fascinating aspect of the metabolism of some species of *Sporomusa* is their ability to obtain energy for growth by decarboxylation of organic acids, even though the associated free energy changes are very small. *Sporomusa malonica* and *S. termitida* can grow by decarboxylation of malonate to acetate +CO_2 (equation 4; Breznak et al., 1988; Dehning et al., 1989).

$$\text{Malonate}^{2-} + H_2O \rightarrow \text{acetate}^- + HCO_3^-$$
$$(\Delta G^{0'} = -17.4 \text{ kJ}) \qquad (4)$$
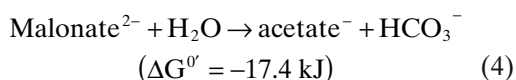
In *S. malonica*, malonate fermentation proceeds by way of malonyCoA, which is decarboxylated by a biotin-containing enzyme (Dehning and Schink, 1994). These two species, as well as *S. acidovorans*, can also grow by decarboxylation of succinate to propionate +CO_2 (equation 5; Breznak et al., 1988; Dehning et al., 1989).

$$\text{Succinate}^2 + H_2O \rightarrow \text{propionate}^- + HCO_3^-$$
$$(\Delta G^{0'} = -20 \text{ kJ}) \qquad (5)$$
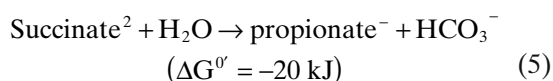
The mechanism by which these decarboxylation reactions couple to ATP formation (a process termed "decarboxylation phosphorylation"; Dimroth and Schink, 1998) is not yet known.

Demethoxylation of methoxylated aromatic compounds can support growth and acetogenesis by *S. termitida* (Breznak et al., 1988), *S. silvacetica* (Kuhner et al., 1997) and *S. ovata* (Stupperich et al., 1996). The *O*-demethylase activity of *S. ovata* grown on 3,4-dimethoxybenzoate was found to have rather broad specificity, being also able to demethylate methoxynaphthols, methoxyfuran and fluoroanisols. Presumably, such substrates will support growth as well. Acrylate groups of methoxylated aromatic compounds are reduced during growth of *S. silvacetica* (Kuhner et al., 1997). However, it is not known whether energy is conserved during such reduction.

Although acetate is the sole or major product from most substrates, propionate is the major product formed from succinate by some species (see eq. 5). In addition, *S. malonica* forms propionate and acetate from malate and fumarate (Dehning et al., 1989), whereas *S. silvacetica* dismutates fumarate to succinate and acetate (Kuhner et al., 1997). *Sporomusa malonica* and *S. paucivorans* form acetate + propionate during fermentation of propanol or 1,2-propanediol, and butyrate + acetate during the fermentation of butanol (Hermann et al., 1987; Dehning et al., 1989), and so resemble *Acetobacterium carbinolicum* (Eichler and Schink, 1984). In addition, *S. paucivorans* forms isobutyrate + acetate from isobutanol (Hermann et al., 1987).

Ecology

The distribution of *Sporomusa* in anoxic habitats seems to be fairly wide. However, our understanding of their ecology is still fragmentary. Like that of other H_2/CO_2-utilizing homoacetogens, their threshold for H_2 is relatively high (430 and 830 parts per million by volume (ppmv) for *S. acidovorans* and *S. termitida*, respectively; Cord-Ruwisch et al., 1988), implying that they are unlikely to be effective competitors for H_2 in the presence of H_2-utilizing methanogens, sulfidogens or denitrifiers. Rather, it would appear to be their ability as a group to use a wide range of substrates that may grant them significant membership in decomposer communities, augmented by their ability to utilize H_2 chemoautotrophically, or mixotrophically (Breznak and Blum, 1991), when competition for this substrate is not severe. Hence, a more likely role for them in

anaerobic microbial communities is as ferment-
ers of small molecules, partnering with methano-
gens and/or sulfidogens through interspecies
transfer of H_2. Indeed, by interspecies transfer of
H_2 during growth on methanol, *Sporomusa* could
support the growth of H_2-consuming methano-
gens and sulfidogens unable to use methanol on
their own (Cord-Ruwisch and Ollivier, 1986;
Heijthuijsen and Hansen, 1986; Hermann et al.,
1987). Under such conditions, a greater fraction
of the methanol was completely oxidized to CO_2
by the *Sporomusa* and proportionally less (or
no) acetate was formed by the coculture. Inter-
species transfer of H_2 to methanogens has also
been observed for *S. paucivorans* during glycerol
fermentation (Hermann et al., 1987). As H_2-
utilizing strains of *Sporomusa* also produce a
small amount of formate during chemoauto-
totrophic growth, interspecies transfer of for-
mate is also possible (Peters et al., 1999).
Syntrophic utilization of organic substrates with
methanogens and sulfidogens may account for
the significant populations of *Sporomusa* in rice
paddy soils (nearly 10^9 cells per g dry soil; Rosen-
crantz et al., 1999) and lake sediments (ca. 105
cells/ml; Sass et al., 1997; Sass et al., 1998).

   Sporomusa also can be found in habitats that
are not strictly, or entirely, anoxic. For example,
S. silvacetica was isolated from well-drained,
aggregated forest soil, a habitat subject to fluctu-
ations in aeration and redox potential. Presum-
ably, its microhabitat in soil consists of anoxic or
microoxic pockets present in soil crumbs (Kuh-
ner et al., 1997). Preliminary studies of the O_2
tolerance of *S. silvacetica* and other homoaceto-
gens revealed that 0.5–1% O_2 in the gas phase of
non-chemically-reduced media had little or no
effect on growth rates or biomass yield. The O_2
was consumed by the acetogens under such con-
ditons, although it is not yet known whether such
O_2 consumption is coupled to energy conserva-
tion (Karnholz et al., 2000). The tolerance to O_2
of *S. silvacetica* and other homoacetogens helps
account for the H_2-consuming, acetogenic capac-
ity of soils (see Kuhner et al., 1997, and refer-
ences therein). Similarly, *S. termitida* was isolated
from hindguts of wood-eating termites, another
habitat consisting of oxic and anoxic zones
(Brune et al., 1995; Ebert and Brune, 1997),
wherein it was felt to contribute to acetogenesis
from H_2+CO_2 and, hence, termite nutrition
(Breznak et al., 1988; Breznak, 1994a).

   Recognition of the wide distribution of *Sporo-
musa*, and the current availability of 16S rRNA
sequences of representatives of all known spe-
cies of the genus, will hopefully prompt a more
comprehensive examination of the ecology of
this group of bacteria, an effort that should be
facilitated by the use of rRNA sequence-based
molecular tools.

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CHAPTER 1.3.5

The Family Lachnospiraceae, Including the Genera *Butyrivibrio*, *Lachnospira* and *Roseburia*

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Butyrivibrio

Introduction

Members of the genus *Butyrivibrio* are non-spore forming, anaerobic, motile, curved rod-shaped bacteria that are commonly isolated from the gastrointestinal tract of mammals. The Gram-negative staining of these organisms in no way reflects the true nature of the cell wall structure. Chemical and electron microscopic analyses of *Butyrivibrio* cells have revealed a thin, Gram-positive ultrastructure (Cheng and Costerton, 1977; Hespell et al., 1993). The genus is composed of two species, *B. fibrisolvens* and *B. crossotus*. Ruminal butyrivibrios characteristically produce butyric acid and degrade plant fibers such as xylans. Hence, the name *Butyrivibrio fibrisolvens* is quite descriptive (Bryant and Small, 1956a). *Butyrivibrio* strains isolated from human fecal material differ in substrate utilization and flagellar arrangement from *B. fibrisolvens*. These strains have been designated as *B. crossotus* to reflect the presence of "tasseled," multiple flagella (Moore et al., 1976). Phylogenetic (DNA–DNA hybridization and 16S rDNA gene sequence) analyses indicate *B. fibrisolvens* strains are a genetically heterologous group of organisms, likely comprised of several species and numerous unrelated strains (Table 1; Mannarelli, 1988; Forster et al., 1996; Mannarelli et al., 1990b; Willems et al., 1995). However, these strains obviously have enough phenotypic similarities to be used to presumptively identify new isolates as *B. fibrisolvens*. These findings are not entirely surprising as it has been known for some time that *B. fibrisolvens* strains differ greatly in serological properties (Margherita and Hungate, 1963; Margherita et al., 1964; Hazlewood, et al., 1986) and in nutritional properties (Bryant and Small, 1956a; Bryant and Robinson, 1962).

Phylogeny

The *Butyrivibrio* are phylogenetically placed within Cluster XIVa of the *Clostridium* subphy-lum, as defined by Collins et al., 1994. Cluster XIVa contains a diverse array of Gram-positive bacteria including members of the *Clostridium*, *Coprococcus*, *Lachnospira*, *Eubacterium*, *Ruminococcus*, *Syntrophococcus*, *Roseburia* and "*Acetitomaculum*." The *Butyrivibrio* belong to three phylogenetically distinct groups (Fig. 1) within this cluster (Forster et al., 1996; Willems et al., 1995). Although the majority of the *Butyrivibrio* will stain Gram-negative, the phylogenetic placement of the group among the low mol% GC content Gram-positive bacteria supports studies that demonstrated a Gram-positive type cell wall structure (Cheng and Costerton, 1977; Hespell et al., 1993).

The strains of *B. fibrisolvens* designated to be in group I are those strains that cluster with the type strain, D1 (ATCC 19171). The strains within this group have 16S rDNA similarities of ≥92% and include the recently described *C. proteoclasticum* (Attwood et al., 1998). Strains clustering in group II have similarities of ≥96% and include *Pseudobutyrivibrio ruminis*. A distinct subgroup consisting of strains OB189, OB156, OB157 and OB192 appears in Group II (designated group IIb). All of the strains in this subgroup were isolated from the rumen of a wild white-tailed deer and have not been detected in significant numbers in domesticated ruminants. Similarities between groups I and II range from 86.8% to 89.9%. *B. crossotus* (group III) has similarities to groups I and II of approximately 90%. There are more than 31 distinct rRNA types within the *Butyrivibrio* (Table 1), and this number will increase as the 16S rDNA sequence of more strains are determined. The levels of rRNA similarity within and between the groups of *Butyrivibrio* indicate that many of these strains, especially those within group I, represent different species within the *Butyrivibrio* genus, and that groups II and III could be considered to be genera in their own right.

In general, the 16S rRNA phylogenetic analysis of the *Butyrivibrio* have supported the DNA:DNA homology analysis of (Mannarelli, 1988) and (Mannarelli, et al., 1990b). However, there are four *Butyrivibrio* strains for which

Table 1. Current groupings of *Butyrivibrio* based on 16S rRNA analysis and corresponding DNA homology and rRNA type designations.

Strains[a]	Mannarelli DNA homology group[b]	Willems 16S rRNA type[c]
Group I		
1-D1 (ATCC 19171)	—	1
2-ACTF2	8	—
3-GS114	8	—
4-D16F	—	—
5-OB235	—	—
6-LM8/1B	—	—
7-A38	—	2
8-Bu 42 (NCDO2434)	—	5
9-NCDO2435	—	6
10-*C. proteoclasticum*	—	—
11-NCDO2398 (B835 Willems)	—	3
12-NCDO2432 (Ce65)	—	4
13-Bu43, Ce64	—	7
14-OB251	—	—
15-E46A	5	—
16-X10C34, OB244	—	—
17-H4a	—	—
Group IIa		
18-H10b	—	—
19-Bu49, UC12254, H17c, (VV1)	1 (6)	8
20-NCDO2397	—	10
21-OR35, OR36, OR37, OR38	—	—
22-T63	—	—
23-CF3, NCDO2399	2	11
24-*P. ruminis*	—	—
25-M3.8	—	—
26-BspS42	—	—
27-Bsp45, AR27	—	—
28-OR78, OR77, NOR37, AR73,	3	9
28-OB236, OR79, OR76, E14,	3	9
28-GS111, GS113, IL6-31	3	9
28-B835 (Russell)	3	9
Group IIb		
29-OB189	—	—
30-OB156, OB157, OB192	—	—
Group III		
31-*B. crossotus*	6	12

[a]Strains listed on the same line or with the same number designation have at least 99.4% 16S rRNA similarity.
[b]Mannarelli et al., 1990b.
[c]Willems et al., 1996.

these analyses disagree. The 16S rRNA sequence of strain B835 (NCDO 2398) was initially determined to be related to group I *Butyrivibrios* (Willems et al., 1995), but the DNA:DNA determinations of Mannarelli, 1988 indicated it was closely related to NOR37 and the group II butyrivibrios. Recently Diez-Gonzalez et al., 1999 determined the 16S rRNA sequence of strain B835 used by Mannarelli, 1988 and found that it was indeed closely related to NOR37. The authors suggest that two unrelated isolates have the same B835 strain designation. The results of (Mannarelli et al., 1990b) also indicate that strains VV1–VV5 cluster within the *B. crossotus* DNA homology group 6. However, 16S rRNA data indicate that strain VV1 has an identical 5' 16S rDNA sequence to the group II strains Bu49, H17c and UC12254 (R.J. Forster, unpublished ovservation). Some confusion also may exist between strains LM8/1B and M3.8 because the 16S rRNA-based phylogeny places these strains in group I and II, respectively, whereas the DNA:DNA homology analysis produces the opposite result. Strains LM8/1B, M3.8 and the VV1–VV5 strains will need to be reexamined to resolve these inconsistencies.

As discussed throughout this chapter, the *Butyrivibrio* are a heterofermentative group of bacteria. When examined together, the fermentation patterns of the various strains of *Butyriv-*
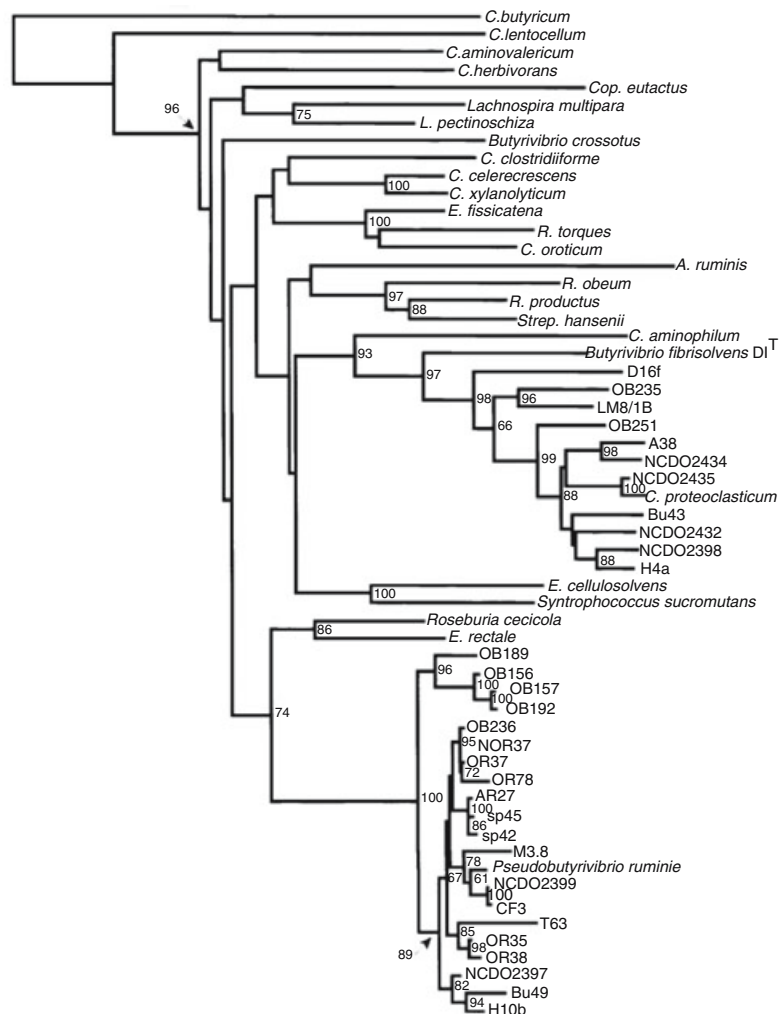
Fig. 1. Phylogenetic tree for *Butyrivibrio*, *Lachnospira*, *Roseburia* and related bacteria. The tree was reconstructed using a neighbor-joining method and the two-parameter model of Kimura as implemented in the PHYLO-WIN program (Galtier et al., 1996). Bootstrap values above 90% are indicated at the branch points, expressed as the percentage from 1000 resampled trees.

ibrio do not follow a phylogenetically coherent pattern. Differentiating *Butyrivibrio* strains on a phenotypic basis has been problematic. This is exemplified by the description of *Pseudobutyrivibrio ruminis* (van Gylswyk et al., 1996) as a bacterium that is non-xylanolytic, non-amylolytic and non-proteolytic. The description of this genus was based on a single isolate, without comparison to the broad range of *Butyrivibrio* strains in current culture collections. Indeed, strains of *B. fibrisolvens* such as CF3 and OR35, which share greater than 99% 16S rRNA sequence homology to *P. ruminis*, are highly xylanolytic, ferment starch and may or may not hydrolyze gelatin. The description of *Pseudobutyrivibrio* thus is not consistent with the phylogenetic classification of the group II *Butyrivibrio*. The observation that butyrate kinase activity is present in group I strains and is absent in group II strains, and conversely that butyryl CoA/acetyl CoA transferase is absent in group I strains and present in group II strains (Diez-Gonzalez et al.,

1999), may lead to a phylogenetically and phenotypically accurate description of these two groups. More strains will need to be examined to determine if these different patterns of butyrate production are indeed consistent between the two main groups of *Butyrivibrio*.

Habitat

Butyrivibrio spp. are common inhabitants of the rumina of cattle and other ruminants (e.g., sheep, goats, reindeer) where they are generally among the more numerous bacterial species present under a variety of different feeding regimes (Stewart and Bryant, 1988; Varel and Dehority, 1989; Dehority, 1986; Dehority and Grubb, 1977; Orpin et al., 1985; Olsen et al., 1997). They also are found in the cecal and fecal contents collected from these animals (Mathieson et al., 1987; Lewis and Dehority, 1985). In addition to ruminants, *Butyrivibrio* spp. are also present in the gastrointestinal tract of a variety of other

mammals and have been isolated from feces or cecal contents of rabbits, horses, humans and swine (Brown and Moore, 1960; Moore et al., 1987; Rumney et al., 1995). *Butyrivibrio* spp. have been isolated from animals in numerous geographical areas throughout the world. It also should be noted that butyrivibrios can occur in environments outside of animals, and strains have been isolated from Napier grass-fed anaerobic digestors (Sewell et al., 1988).

Isolation

BUTYRIVIBRIO FIBRISOLVENS *Butyrivibrio fibrisolvens* strains are extremely versatile and have been isolated using a variety of culture media and growth conditions, however, no definitive selective isolation or enrichment procedures have been published. Strains of *B. fibrisolvens* and *Prevotella ruminicola* are the predominant isolates obtained from ruminal contents when xylan is the only added carbohydrate source provided in the isolation medium (Dehority, 1966; Van der Toorn et al., 1985). *Butyrivibrio* strains also are frequently isolated from ruminal (Shane et al., 1969) or cecal (Lewis and Dehority, 1985) contents when media containing ball-milled or finely ground cellulose are used. With these cellulose-containing media, colonies of butyrivibrios often are surrounded by weak zones of cellulose degradation compared to the extensive clearing produced by cellulolytic cocci (e.g., *Ruminococcus* species) or by nonmotile, pleomorphic rods (e.g., *Fibrobacter succinogenes*). *Prevotella* and *Butyrivibrio* strains constitute the majority of bacterial isolates obtained from ruminal contents when media containing plant saponins are used (Gutierrez et al., 1959). *B. fibrisolvens* strains are resistant to high levels (30 to 500 μg per ml) of nalidixic acid (Hespell et al., 1993), but most strains are quite sensitive to many antibiotics, especially those that affect cell wall synthesis (e.g., penicillin, bacitracin; Fulghum et al., 1968; Hespell et al., 1993). Since all well-characterized *B. fibrisolvens* are uniformly xylanolytic, a selective medium for *B. fibrisolvens* strains might be constructed with xylan as the sole carbohydrate source and nalidixic acid.

BUTYRIVIBRIO CROSSOTUS *B. crossotus* has been isolated from human fecal and rectal contents using nonselective, rumen fluid-glucose-cellobiose medium (Holdeman et al., 1976; Moore et al., 1976). More selective isolation media or enrichment procedures for this species are not known. Although *B. crossotus* is less versatile than *B. fibrisolvens*, the type strain of this species is also resistant to nalidixic acid, which may prove useful in the construction of selective media for this organism (Hespell et al., 1993).

Identification

BUTYRIVIBRIO FIBRISOLVENS Many *B. fibrisolvens* strains produce surface colonies that are 2 to 4 mm in diameter, entire, slightly convex, translucent, and light tan to white in color, when grown on complex carbohydrate agar-containing media (Bryant and Small, 1956a; Bryant, 1986a). Some strains produce rough colonies that have filamentous edges and might represent strains that produce little extracellular polysaccharide material. In contrast, strains such as the CF types produce large amounts of extracellular polysaccharide, the colonies are quite mucoid and, gas bubbles can be seen to emanate from the colonies. Subsurface colonies of most strains are lenticular to "Y" shaped, and double lens-shaped colonies are not uncommon. In cellulose-containing media, the cellulose-digesting strains are surrounded by a zone of cellulose degradation, variable both in zone size and extent of digestion (usually weak). Similar observations also occur when media containing natural xylans are used. However, if a dyed xylan (e.g., Remazol brilliant blue xylan) is used, large clear zones surround the colonies of all strains. Most strains grow rapidly in broth cultures and produce flocculent sediment, whereas some strains produce granular sediment that adheres to glass surfaces. Cultures of these latter strains and those that produce large amounts of extracellular polysaccharides can become quite thick and viscous when the media contain large amounts (≥0.5%) of the energy source.

The classical appearance of *B. fibrisolvens* is a small, motile, slightly curved rod (0.4 to 0.6 mm × 2 to 5 mm long) with tapered and rounded ends (Fig. 2A, B). However, there is considerable variation amongst strains. The cells of some strains are almost spindle-shaped, while cells of other strains are quite curved and can form helices composed of two to four cells.

The cells show translational motility characterized by rapid or intense vibrating movement. Motility is by means of monotrichous flagellation, with the flagellum attached subterminally. Although often only a few cells in a wet mount preparation may show motility, truly nonmotile strains have not been well documented. The lack of motility in an isolated strain is often due to cultural conditions employed. Bryant and Small, 1956a reported that 15 strains were nonmotile when grown in a ruminal fluid-glucose-cellobiose medium, but all were motile if cellobiose was deleted and the glucose concentration decreased. Growth of most strains is rapid (doubling times of 2 h or less) and much acid production occurs. Low pH due to acid production may inhibit motility and also inhibits growth (Gill and King, 1958; Russell and Dombrowski, 1980;
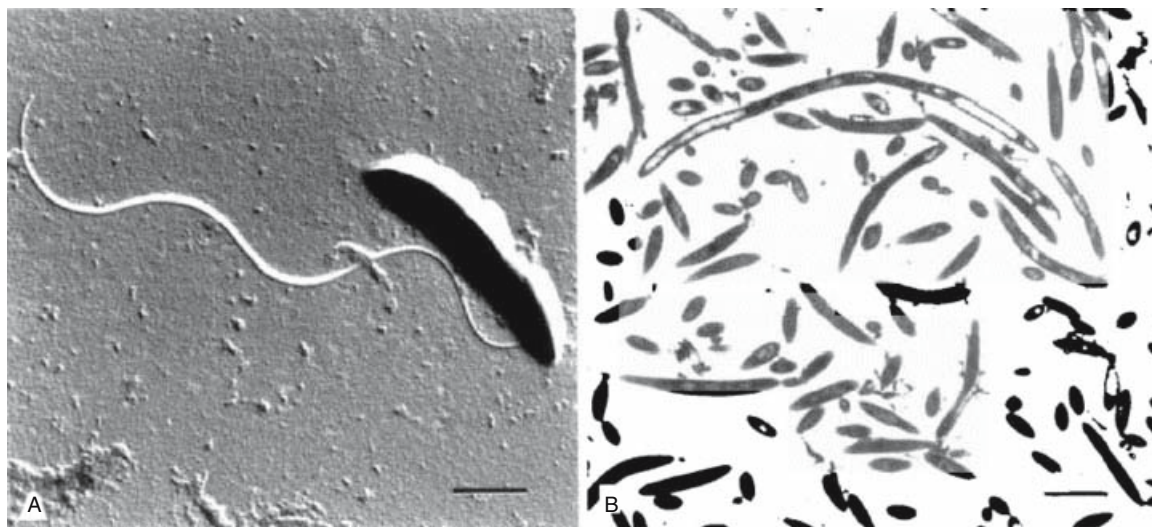
Fig. 2. Electron micrographs of *B. fibrisolvens* 113. (A) Whole cell, platinum-shadowed. Bar = 0.5 μm. (B) Thin section of cells, showing filamentous aspects of attached cells after cross-wall formation. Bar = 1.0 μm. (From Sewell et al., 1988.)

Therion et al., 1982). Because of these effects, cell characteristics and motility should be monitored in young cultures and in media with a low amount of added energy source.

The initial study of *Butyrivibrio* (Bryant and Small, 1956a) and many subsequent studies (Brown and Moore, 1960; Cheng et al., 1969; Shane et al., 1969; Sewell et al., 1988) have consistently reported that *Butyrivibrio* strains appear as Gram-negative cells when stained by conventional procedures and viewed by light microscopy. However, *B. fibrisolvens* strains 49, NOR37, and 1L631 were found to possess lipoteichoic acids, which are present only in Gram-positive bacteria (Sharpe et al., 1975; Hewett, et al., 1976). Later, Cheng and Costerton, 1977 established by electron microscopic observations that *B. fibrisolvens* strain D1 had a Gram-positive cell wall structure. The peptidoglycan layer, however, was very thin (12 to 15 nm) compared to normal Gram-positive bacteria (30 to 50 nm), and it was thought that the Gram-negative staining of *Butyrivibrio* sp. might be based on the thinness of the peptidoglycan. A comprehensive examination of the peptidoglycans from strains of *B. fibrisolvens* (Hespell et al., 1993) indicate the peptidoglycans are of similar composition and low degree of crosslinking: a modified meso-diaminopimelic acid direct-link peptidoglycan as described by Schleifer and Kandler, 1972. Cell wall preparations also contained unusually high concentrations of glucosamine. Electron microscopic examination of cells in this study also confirmed the Gram-positive nature of *Butyrivibrio* cell walls. Consistent with this cell wall type, all *B. fibrisolvens* are quite sensitive to penicillin or bacitracin, but all are resistant to nalidixic acid.

In contrast to many other species of ruminal bacteria, any given *B. fibrisolvens* strain is capable of fermenting a wide variety of carbohydrates. All strains are xylanolytic. The majority of strains ferment starch and pectin. Some strains weakly degrade cellulose. Though it was thought that degradation of rutin was a common trait in *B. fibrisolvens* strains (Hespell and Bryant, 1981), this property is found in only a few strains. Almost all strains can ferment 15 to 20 different soluble carbohydrates and the most commonly used ones include glucose, maltose, sucrose, fructose, cellobiose, xylose, and arabinose. With respect to nitrogen requirements, ammonia can serve as the sole nitrogen source for most *B. fibrisolvens* strains (Bryant and Robinson, 1962). Many strains also can use mixtures of amino acids or more complex nitrogen sources (trypticase, casein hydrolysate, peptone). However, ammonia supports considerably more growth than an equivalent amount of peptide or amino acid nitrogen when these compounds are provided at growth-limiting concentrations (Bryant, 1973). When measured by gelatin liquefaction, many strains appear nonproteolytic. This is probably a misleading conclusion, particularly if high levels (e.g., 10 to 12%) of gelatin are used in the medium for determining activity. A survey of a large number of strains indicated they are proteolytic when this activity is measured by azocasein hydrolysis (Cotta and Hespell, 1986). In fact, constitutive production of protease as well as esterase and lipase (Hespell and O'Bryan-Shah, 1988) activities appears to be very common traits in *B. fibrisolvens* strains.

Butyrivibrio fibrisolvens strains produce large amounts of n-butyric acid during the fermenta-

tion of carbohydrates. Smaller amounts of formate and hydrogen gas also are made by all strains. Many strains produce trace amounts of ethanol, but no strain produces succinate. Although some strains require carbon dioxide to initiate growth, all studied strains produce carbon dioxide (Dehority, 1971). *B. fibrisolvens* strains generally exhibit two major fermentation acid patterns. One type produces large amounts of lactate and removes acetate added to the medium, while the other type produces little or no lactate but substantial amounts of acetate. These fermentation patterns have been used to separate butyrivibrio-like isolates (Shane et al., 1969). Hungate, 1966 proposed another species, *B. alactacidigens*, for those strains that do not produce lactate. More recently, Diez-Gonzalez et al., 1999 found that these phenotypes could be explained by the mechanism of butyrate production used by strains. Acetate utilizing-lactate producing strains (like strain 49) possessed butyryl CoA/ acetylCoA transferase activity, while acetate producing strains (like strain D) used butyrate kinase to produce butyric acid. The presence and absence of these activities in the strains examined were consistent with phylogenetic groupings based on 16S rDNA sequence analyses and may serve as a valuable taxonomic marker.

The production of large amounts of n-butyric acid from the fermentation of carbohydrates, along with cell morphology and Gram-staining, are characteristics that can be used to place a newly isolated strain into the species *B. fibrisolvens* While it seems reasonable that *B. fibrisolvens* strains could be separated on the basis of specific carbohydrate usage into groups that would be consistent with genetic groups (Table 1), sufficient information is not yet available to create this determinative classification. At present, a useful phenotypic trait that allows for placement of newly isolated butyrivibrio-like strains into one of these DNA relatedness groups is neutral sugar composition of the extracellular polysaccharides. These polysaccharides are easily isolated from culture fluids and can be analyzed by well-established techniques (Stack, 1987; Stack, 1988a).

BUTYRIVIBRIO CROSSOTUS In addition to *B. fibrisolvens*, the only other species presently in this genus is *Butyrivibrio crossotus*. This species can be isolated from human rectal or fecal material and differs considerably from *B. fibrisolvens* (Moore et al., 1976). Strains of this bacterium are motile by means of polar to subpolar, lophotrichous flagella. Surface colonies on brain heart infusion agar are quite small (0.2 to 1.0 mm diameter), circular, convex, and translucent. Slightly larger subsurface colonies are produced

and are lenticular in shape. The cell morphology is similar to *B. fibrisolvens* and cells stain Gramnegative. *B. crossotus* has a narrow range of fermentable substrates that include maltose, starch, glycogen, and dextrin. Large amounts of butyrate are made and some hydrogen gas is formed by most strains. Tests for lecithinase, lipase, and ammonia production from peptone are negative. As pointed out by Moore and Holdeman, 1974, *Eubacterium rectale* is part of the normal human fecal flora and many strains are very similar to *Butyrivibrios*.

Cultivation

Members of *Butyrivibrio* are all strictly anaerobic bacteria and appropriate steps should be employed to remove and exclude oxygen from growth medium (e.g., use of the technique of Hungate and modified by Bryant, 1972). The complex, Routine Growth Medium (RGM, Table 2) can be used to grow strains of these bacteria as well as many other species of ruminal bacteria (Hespell et al., 1987). These bacteria can be grown at 37°C, but usually little or no growth takes place at 30 or 45°C. Many *B. fibrisolvens* strains are not nutritionally fastidious and can be grown on a chemically defined medium (Table 2), which has been used in studies of proteolytic activities with these strains (Cotta and Hespell, 1986). Most *B. fibrisolvens* strains can use ammonia as a sole nitrogen source and many strains also can use urea. Peptides in the form of trypticase or other peptones are not required, but often stimulate growth when added to ammonia-containing media (Bryant and Robinson, 1962). The precise vitamin and other growth requirements for most strains of Butyrivibrio have not been determined.

Preservation

Most strains of *Butyrivibrio* can be maintained for long times by storage of cultures in liquid nitrogen or in ultracold freezers (Hespell and Canale-Parola, 1970). Preservation by lyophilization under anaerobic conditions may be possible, and strains obtained from culture collections are provided in this form. Short-term storage (6 to 15 months) is possible by placing glycerol-containing cultures in normal (–20°C) freezers (Teather, 1982a).

Physiology

Butyrivibrio fibrisolvens is quite versatile in the polymeric substrates that it can metabolize, and cellulolytic, xylanolytic, amylolytic, and pectinolytic strains have been isolated. While all characterized strains of *B. fibrisolvens* are highly

Table 2. Media for *Butyrivibrio*, *Lachnospira*, and *Roseburia*.[a]

Ingredient	Amount per 100ml medium	
	Complex—RGM[b]	Defined—*B. fibrisolvens*
Carbohydrate (5%)[c,d]	5.0ml	5.0ml
Trypticase	0.3g	—
Yeast Extract	0.2g	—
Ammonium chloride (5.3%)	1.0ml	1.0ml
Vitamin solution[c,e]	—	1.0ml
IVI VFA solution[f]	3.0ml	3.0ml
Mineral A[g]	4.0ml	4.0ml
Mineral B[h]	4.0ml	4.0ml
Trace minerals[i]	0.1ml	0.1ml
Hemin/naphthoquinone solution[j]	1.0ml	1.0ml
Sodium carbonate solution (8.0%)[c,k]	5.0ml	5.0ml
L-cysteine XHCl (5.0%)[c,d]	1.0ml	1.0ml
Resazurin (0.1%)	0.1ml	0.1ml
Distilled Water	76.0ml	75.0ml

[a]Prepared anaerobically under carbon dioxide gas phase; final pH 6.8.
[b]RGM, routine growth medium.
[c]Prepared separately and added to cooled, autoclaved medium.
[d]Prepared, autoclaved, and stored under a nitrogen gas phase.
[e]Containing per 100ml N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffer (5mM; pH 7.5): biotin, folic acid, Δ-aminobenzoic acid, and cyanocobalamin (2.5mg each); calcium pantothenate, nicotinamide, riboflavin, thiamine hydrochloride, and pyridoxamine (20mg each). The resulting solution is filter sterilized (pore size, 0.22μm) and stored under nitrogen in a dark container.
[f]Prepared by adding 7-ml acetate, 3-ml propionate, 2-ml butyrate, and 0.6-ml each of isobutyrate, 2-methyl-butyrate, isovalerate, and n-valerate to 700ml of 0.2M NaOH. Adjust to pH 7.0 with NaOH and to final volume of 11.
[g]Mineral A = 0.5% K_2HPO_4.
[h]Mineral B = 1.0% KH_2PO_4, 1.2% NaCl, 0.58% $NaSO_4$, 0.16% $CaCl_2X2H_2O$, and 0.25% $MgSO_4X7h_2O$.
[i]Prepared as described by Hespell and Canale-Parola (1970) by adding 25ml of modified Pfennig's metals to 3.75g $CaCl_2X2H_2O$, 12.5g $MgCl_2X6H_2O$, 0.5g $FeSO_4X7H_2O$, and 1.0g Na_4EDTA dissolved in 75ml distilled water. Modified Pfennig's metals are prepared by separately dissolving (heat if needed) each of the following compounds in distilled water and adding (in order) to 11 distilled water: 0.5g $AlCl_3$, 0.25g KI, 0.25g KBr, 0.25g LiCl, 3.5g $MnCl_2X4H_2O$, 5.5g H_3BO_3, 0.5g $ZnCl_2$, 0.5g $CuCl_2X2H_2O$, 0.5g $NiCl_2X6H_2O$, 0.5g $CoCl_2X6H_2O$, 0.15g $SnCl_2X2H_2O$, 0.15g $BaCl_2X2H_2O$, 0.25g $NaMoO_4X2H_2O$, and 0.05g $NaVO_3$. The resultant solution is adjusted to pH 3.0 to 4.0 with HCl and brought to a final volume of 1.81 with distilled water.
[j]Made by dissolving 25mg 1.4-naphthoquinone in 2ml 95% ethanol, adding 48ml distilled water and 10mg hemin dissolved in 50ml of 0.01M NaOH.
[k]Autoclaved separately; prepared, equilibrated, and stored under a carbon dioxide gas phase.

xylanolytic, only about 10% of these strains are capable of good growth on cellulose. A few strains are capable of anaerobically degrading complex heterocyclic compounds of the bioflavonoid type (Cheng et al., 1969; Cheng et al., 1970). Some strains can deacetylate trichothecenes such as T-2 toxin (Westlake et al., 1987). *B. fibrisolvens* is active in the biohydrogenation of fatty acids in the rumen (Harfoot and Hazlewood, 1988) and strains producing phospholipase and galactolipase have been identified (Hazlewood, et al., 1983).

Because of its role in the digestion and metabolism of hemicellulose (xylan) in the rumen, much research has focussed on the xylanolytic activity of *B. fibrisolvens*. Xylan is a complex polysaccharide contained in plant cell walls and is composed of a β 1,4-linked xylose backbone with side groups of arabinose, uronic acids, and other sugars. The xylose residues are also frequently acetylated. As a result, several enzymatic activities are required to hydrolyze this polysaccharide to monosaccharides. *B. fibrisolvens* strains have been identified that produce endo-xylanase, β-xylosidase, arabinofuranosidase, cinnamoyl esterase and acetylxylan esterase (Hespell and O'Bryan-Shah, 1988; Hespell and Whitehead, 1990; Williams and Withers, 1992a; Williams and Withers, 1992b; Lin and Thomson, 1991a; Hespell et al., 1987; Akin et al., 1993). In some cases the genes responsible for these activities have been cloned and analyzed and the proteins purified and characterized (Dalrymple et al., 1996; Thompson, 1993; Sewell et al., 1989; Hespell and O'Bryan, 1992; Utt et al., 1991; Lin and Thomson, 1991b; Mannarelli et al., 1990a; Dalrymple and Swadling, 1997). The production of xylanolytic enzymes is regulated in strains that have been examined. Some evidence exists that these enzymes may be in a large molecular weight assemblage containing a variety of extracellular enzyme activities. *B. fibrisolvens*

also produces amylase, endo-glucanase, pectin esterase, and exopectin lyase activities also important in the digestion and metabolism of plant polysaccharides (Thompson, 1993; Hazlewood et al., 1990; Heinrichova et al., 1985; Wojciechowicz et al., 1982; Cotta, 1988; Rumbak et al., 1991).

Most strains are proteolytic and produce protease activity equal to or greater than that found with other proteolytic ruminal bacteria such as *Ruminobacter amylophilus* or *Prevotella ruminicola* (Cotta and Hespell, 1986; Wallace and Brammall, 1985; Strydom et al., 1986). The most active strains appear to produce extracellular proteolytic activity of the serine protease type. Some low activity strains producing cell-associated, cysteine protease-like activities also have been identified. Regulation of protease activity has not been studied extensively. In strain 49, the activity is constitutively produced, but growth parameters and the availability of complex nitrogen sources modulate the level of activity (Cotta and Hespell, 1986). *B. fibrisolvens* produces peptidases and produces ammonia from peptides and amino acids (Wallace and McKain, 1991; Bladen et al., 1961; Scheifinger et al., 1976). Glutamate dehydrogenase and glutamine sythetase (type GS III) activities have been detected in *B. fibrisolvens* (Goodman and Woods, 1993; Joyner and Baldwin, 1966). Some strains are ureolytic (Wozny et al., 1977). Little is known about control of these activities.

B. fibrisolvens strains ferment a wide array of hexoses and pentoses, and the utilization of carbohydrates is regulated. Although not examined for many strains, substrate utilization patterns suggest the involvement of catabolite regulatory mechanisms (Russell and Baldwin, 1978). However, evidence for the involvement of cAMP or regulatory elements of the phosphoenol pyruvate, phosphotransferase systems is lacking (Cotta et al., 1994; Strobel, 1994; Martin and Russell, 1986). *B. fibrisolvens* strains characteristically produce butyric acid as the main product from fermentation of carbohydrates (Bryant and Small, 1956a; Hespell et al., 1987). The formation of the other fermentation acids varies considerably, depending upon the strain. Shane et al., 1969 subdivided strains of *Butyrivibrio* into two groups. Group 1 strains utilized acetate and made small amounts of formate and large amounts of lactate. Group 2 strains produced acetate and made large amounts of formate and small amounts of lactate. This phenotypic difference is not always clear-cut and can be influenced by culture conditions. Utilization of acetate is stimulated by low availability of carbon dioxide, presence of lactate, and growth-limitation of energy source (Latham and Legakis, 1976; Jarvis et al., 1978). In addition, acetate utilization is affected by the levels of acetate added to the medium as well as by the growth rate of the culture. The levels of acetate can markedly affect the production of lactate by certain strains. The utilized acetate is primarily incorporated into butyrate on almost an equal molar basis with acetate formed from the fermented carbohydrate source.

Biochemical and enzymatic studies with *B. fibrisolvens* have not been extensive. The key enzymes of the Embden-Meyerhof pathway plus lactate dehydrogenase, phosphotransacetylase, and acetate kinase have been detected at high levels (Joyner and Baldwin, 1966; Kistner and Kotze, 1973; Kotze and Kistner, 1974). Based on studies employing radiolabeled substrates, pyruvate synthase and pyruvate-formate lyase activities are present in *B. fibrisolvens* (van Gylswyk, 1976). The formation of butyryl-CoA from acetyl-CoA involves the sequential actions of thiolase, β-hydroxy butyryl-CoA dehydrogenase, crotonase, and crotonyl-CoA reductase (Miller and Jensel, 1979). Butyryl-CoA is converted to butyrate by phosphotransbutyrylase and butyrate kinase or via butyryl CoA/acetyl CoA transferase reactions (Diez-Gonzalez et al., 1999). In regard to biosynthetic metabolism, oxaloacetate is formed by the action of phosphoenolpyruvate carboxykinase (van Gylswyk, 1979). Glutamate, succinate, malate, and isocitrate dehydrogenases appear to be present (Joyner and Baldwin, 1966).

Genetics

The isolation of a large, 200-kDa plasmid from *B. fibrisolvens* (Teather, 1982b) was the first demonstration that strains of *Butyrivibrio* contained extrachromosomal elements. A search for smaller plasmids that could prove useful for the development of genetic transformation systems resulted in the isolation of a 2.8-kb plasmid, pOM1, from *B. fibrisolvens* 49 (Mann et al., 1986). An additional native plasmid, pBF1, was cloned and characterized from *B. fibrisolvens* AR10 (Ware et al., 1992). The first full sequence of a plasmid from a rumen bacterium was obtained from *B. fibrisolvens* OB156 (Hefford et al., 1993). The plasmid, pRJF1, was determined to be 2.6 kb and contained a complex series of direct and inverted repeats, reminiscent of theta-replication regions of other Gram-positive plasmids. The plasmid encoded two open reading frames (ORF's), which had limited homology to known sequences, and a unique *Hind*III site within a noncoding stretch of DNA bounded by a 79-bp inverted repeat. This unique *Hind*III site was later used to combine the *E. coli* vector pUC118 and an erythromycin resistance gene from pAMβ1 with pRJF1, to produce a stable

rumen bacterial shuttle vector (pBHerm) to transform *B. fibrisolvens* by electroporation (Beard et al., 1995).

In a further effort to develop transformation systems, pOM1 was sequenced, and a stable shuttle vector (pSMerm) developed by combining the same *E. coli* replication elements and erythromycin-resistance gene introduced into pRJF1 (Hefford et al., 1997). Plasmid pOM1 was determined to replicate by a rolling circle mechanism, and pSMerm could be transformed simultaneously into *B. fibrisolvens* OB156 with pBHerm. An additional plasmid transformation system has been developed using pUC18 and pAMβ1 with the native plasmid pRJF2 (Kobayashi et al., 1995), isolated from *B. fibrisolvens* OB 157. Plasmids pRJF1 and pRJF2 are highly homologous, and differ mainly in sequence and length in the noncoding region between the conserved 79-bp repeats. Optimal electroporation conditions for these plasmids have been published (Kobayashi et al., 1997).

The first successful introduction of plasmid DNA into *B. fibrisolvens* was accomplished by conjugation using the transposon Tn*916* and the self-mobilizing plasmid pAMβ1 (Hespell and Whitehead, 1991). Subsequently, conjugation with pUB110 from *S. aureus* (Clark et al., 1994) into *Butyrivibrio* was also achieved. Stable transformation of *B. fibrisolvens* also has been demonstrated using electroporation with the *E. coli/B. subtilis* shuttle vector pBS42 (Whitehead, 1992). Although a number of *Butyrivibrio* strains have been successfully transformed with the systems described above, the majority of strains are resistant to transformation by electroporation. Restriction-modification systems have been characterized in *Butyrivibrio* (Mohn and Teather, 1995) and may account for this lack of success.

A number of genes cloned from strains of *B. fibrisolvens* play a role in polysaccharide degradation. These genes include an endoglucanase (*endI*) from H17c (Berger et al., 1990), a β-D-xylosidase/α-L-arabinofuranosidase (*xylB*) from GS113 (Sewell et al., 1989), a xylanase (*xynA*) from strain 49 (Mannarelli et al., 1990a), an endoglucanase (*celA*) from A46 (Hazlewood et al., 1990), a β-glucosidase (*bglA*; Lin et al., 1990), cellodextrinase (*cedI*; Berger et al., 1990), and a xylanase (*xynB*; Lin and Thomson, 1991b) from strain H17c as well as two cinnamoyl ester hydrolases (*cinA* and *cinB*) from strain E14 (Dalrymple et al., 1996; Dalrymple and Swadling, 1997). An investigation of the distribution and evolutionary relationships of the *xynA* and *xynB* genes from *Butyrivibrio* revealed that the *xynB* gene was present in three of the seven group I *Butyrivibrio* strains examined (Dalrymple et al., 1999). However the *xynA* gene was found to con-

sist of two subfamilies, each of which was present in group I and group II strains. It was noted that 5 of the 6 best xylan-degrading strains of *Butyrivibrio* contained a member of *xynA* subfamily A.

The expression and secretion of an introduced fungal xylanase in *B. fibrisolvens* OB156 was improved by manipulating promoter sequences (Xue et al., 1997). Successful expression of the xylanase was found with a promoter sequence from *B. fibrisolvens* strain 49 but strain OB156 did not efficiently recognize E. coli promoter sequences. The secretion of the xylanase also was improved significantly when a signal sequence from *B. fibrisolvens* H17c was manipulated to increase its hydrophobicity. Further increases in the efficiency of gene expression in *Butyrivibrio* will be dependent upon detailed analysis of *Butyrivibrio* promoter sequences. Recently a promoter-rescue plasmid for *B. fibrisolvens* has been described (Beard et al., in press), which will aid in this important area of research.

Other genes cloned from *B. fibrisolvens* H17c include an α-amylase (*amyA*) (Rumbak et al., 1991a), a 1,4-α-glycosyltransferase (*glgB*; Rumbak et al., 1991b) and a glutamine synthase gene (Goodman and Woods, 1993). The structural gene (*bvi79A*) encoding a bacteriocin from *B. fibrisolvens* strain OR79 was cloned and characterized (Kalmokoff et al., 1999). Along with the bacteriocin encoding gene, the cloned fragment was found to contain 4 other ORFs with significant homology to genes involved in antibiotic production or immunity. The bacteriocin gene encoded a prepeptide of 47 amino acids and was processed into a mature bacteriocin of 25 amino acids.

Ecology

Butyrivibrio fibrisolvens strains are invariably found in the rumen of animals fed a wide range of diets, but these bacteria usually are in considerable numbers with animals fed diets high in forage materials that are generally poorly digested (Thorley et al., 1968; Shane et al., 1969). All *B. fibrisolvens* strains attack the hemicellulose (xylan) portion of plant cell walls, and many also can hydrolyze other plant polysaccharides like starch and pectin (Hespell et al., 1987). Some strains also can degrade cellulose. Most strains are proteolytic (Cotta and Hespell, 1986). *Butyrivibrio* strains have been identified that are active in specialized activities that occur in the rumen including fatty acid biohydrogenation, bioflavanoid degradation (e.g., rutin), and mycotoxin modifications (Harfoot and Hazlewood, 1988; Westlake et al., 1987; Cheng et al., 1969). Given this wide biochemical diversity of *Butyrivibrio* strains, these bacteria are involved in many important ecological roles in the rumen

and probably also in the cecum and large intestine of many mammals.

Although not as effective at fiber digestion as species of *Ruminococcus* or *Fibrobacter*, many *Butyrivibrio* strains are capable of solubilizing about 20–45% of the entire plant cell wall (Morris and van Gylswyk, 1980). During the degradation of xylan, solubilization occurs at a faster rate than utilization of the breakdown products (Dehority, 1967). In pure culture, these breakdown products have been identified as xylooligosaccharides and can serve as substrates for the growth of non-xylanolytic, oligosaccharide-fermenting bacteria (Cotta and Zeltwanger, 1995; Hespell and Cotta, 1995). A similar phenomenon has been demonstrated for the degradation of starch by *B. fibrisolvens* (Cotta, 1992). Improved utilization of polysaccharides can be observed when *B. fibrisolvens* is cocultivated with other ruminal bacteria, but synergistic attack on polysaccharides was not apparent. Such cross feeding between polysaccharide-hydrolyzing and simple sugar-fermenting species are thought to be an important ecological interaction among species present in the rumen environment.

B. fibrisolvens is one of the most numerous protein-degrading species of bacteria isolated from the rumina of animals on various diets (Blackburn and Hobson, 1962; Fulghum and Moore, 1963; Hazlewood et al., 1983). Most strains are proteolytic and produce protease activity equal to or greater than that found with other proteolytic ruminal bacteria such as *Ruminobacter amylophilus* or *Prevotella ruminicola*. *B. fibrisolvens* produces peptidases and was identified by Bladen et al., 1961; Scheifinger et al., 1976) as an important ammonia-producing rumen organism, although it is much less active in this regard than some of the obligate amino acid fermenting species isolated more recently (Russell et al., 1988; Attwood et al., 1998; Paster et al., 1993). It has been hypothesized that this proteolytic activity, in addition to providing nitrogen for growth, may be important in making starch contained in granules embedded in protein matrices more available (McAllister et al., 1990; McAllister et al., 1993).

Recently it was discovered that *B. fibrisolvens* strains commonly produce compounds (Kalmokoff et al., 1997; Kalmokoff et al., 1999) that inhibit the growth of a number of different bacterial species including other ruminal bacteria—even other strains of *B. fibrisolvens* themselves. The bacteriocins, Butyrivibriocin AR10 and OR79A, were isolated and characterized. The effect of bacteriocin activity on *B. fibrisolvens*' ecology is unknown but could be to enhance the ability of this organism to compete with other species in the highly competitive rumen environment.

Applications

At present, there are no biotechnological applications of *Butyrivibrio* sp. Because *B. fibrisolvens* produces a large array of extracellular polysaccharide-degrading enzymes, these organisms may represent a rich source of these enzymes and of genes for cloning. A recombinant yeast strain was constructed that contained an endo-glucanase from *B. fibrisolvens* to attempt to exploit this polysaccharide-degrading capacity (van Rensburg et al., 1997; van Rensburg et al., 1994). Applications to biomass fermentors also may be possible because *B. fibrisolvens* is a major portion of the xylanolytic isolates obtained from napier grass-fed anaerobic digesters (Sewell et al., 1988). *B. fibrisolvens* strains also produce extracellular polysaccharides (EPS) containing unusual sugars (Stack, 1988a; Stack and Weisleder, 1990; Stack et al., 1988b; Stack and Weisleder, 1990). The structures of some of these EPS have been elucidated and the potential for industrial use of one of these has been investigated (Ha et al., 1991; Ferreira et al., 1995; Ferreira et al., 1997).

Most interest in the commercial exploitation of *B. fibrisolvens* lies in its function in the rumen. Because it is a numerically important species under a variety of feeding conditions, *B. fibrisolvens* has been identified as a potential target for genetic manipulation. The goal of such an approach is to develop inocula that can be used to improve the digestion of feeds in agriculturally important ruminant livestock species, such as cattle and sheep. Towards this end a recombinant strain of *B. fibrisolvens* was developed that expresses dehalogenase activity and thereby the ability to detoxify the plant toxin fluoroacetate (Gregg, 1995; Gregg et al., 1994). The recombinant strain was successfully introduced into the rumen of sheep conferring resistance to those animals consuming this toxic agent. This strain was not developed further because of concerns over environmental release of this recombinant organism.

Lachnospira

Introduction

Lachnospira sp. are Gram-positive, nonsporeforming, obligately anaerobic, motile, rod-shaped, pectin-fermenting bacteria isolated from the intestinal tracts of animals. The genus is composed of two species, *L. multipara* and *L. pectinoschiza*. Originally isolated as curved or helical rods that formed filamentous or "wooly" colonies, this genus was given the name *Lachnospira*, or wooly hair coil, to emphasize this unique characteristic (Bryant and Small, 1956b). These orig-

inal isolates from the rumen also formed many fermentation products, thus the name *L. multiparus* (now *L. multipara*). More recently a second species, *L. pectinoschiza*, was isolated from cecal contents and feces of pigs (Cornick et al., 1994). Although this species does not form the characteristic "wooly" colonies, analysis of the 16S rRNA sequence indicated a close relationship with *L. multipara* and therefore this organism was placed in the same genus. The name *L. pectinoschiza* given to this species identifies its "pectin splitting" activity.

Phylogeny

The *Lachnospira* are placed phylogenetically within cluster XIVa of the *Clostridium* subphylum, as defined by Collins et al., 1994. Cluster XIVa contains a diverse array of Gram-positive bacteria including members of the genera *Clostridium*, *Coprococcus*, *Eubacterium*, *Ruminococcus*, *Syntrophococcus*, *Roseburia*, "*Acetitomaculum*" and *Butyrivibrio*. The *Lachnospira* form a monophyletic group within this cluster with *L. multipara* and *L. pectinoschiza* grouping together.

The phylogenetic relationship of the two species of *Lachnospira*, as determined by 16S rRNA analysis (Cornick et al., 1994), agrees with the results of DNA-DNA hybridization and extracellular polysaccharide analysis (Mannarelli et al., 1990b). *L. multipara* has a similarity to *L. pectinoschiza* of approximately 94% and to *C. celecrescens*, *E. rectale* and *R. cecicola* of 90 to 91%. *L. pectinoschiza* is more closely related to *R. cecicola*, with a similarity of 93%. (See Fig. 1.)

Habitat

Lachnospiras are normal inhabitants of the rumen and also have been isolated from the gastrointestinal tracts of pigs, reindeer, and iguanas (Stewart and Bryant, 1988; Cornick et al., 1994; McBee and McBee, 1982; Mathieson et al., 1987). Although generally present in low numbers (e.g., 0.1% of total bacteria) in rumen contents of animals fed many diets, *Lachnospira multipara* can be present in high numbers when ruminants are fed diets rich in pectin (Bryant et al., 1960). *Lachnospira pectinoschiza* was isolated as a "pectinophile" from the cecum of the pig and also could be recovered from feces of these animals. It is likely that *Lachnospira* sp. will be found in the gastrointestinal tracts of other animals, when adequate selective isolation procedures or specific detection methods are developed for these organisms.

Isolation

LACHNOSPIRA MULTIPARA All *Lachnospira multipara* strains are pectinolytic and have been isolated from ruminal contents with media containing pectin as the only added energy source (Dehority, 1969). These media are only partially selective as colonies of other pectin-fermenting bacteria such as *Butyrivibrio fibrisolvens* and *Prevotella ruminicola* are also obtained. More selective isolation media or enrichment procedures for *L. multipara* are not known. The type strain is resistant to kanamycin and neomycin, so it might be possible to devise a selective medium containing pectin and these antibiotics (Wang et al., 1969). A similar approach using starch and kanamycin was successfully applied to the selective isolation *Streptococcus bovis* from the rumen (D. Odelson, personal communication). *L. multipara* has few nutritional requirements (Bryant and Robinson, 1962), which might also be incorporated into the development of more selective isolation procedure.

Lachnospira pectinoschiza Cornick et al. (1994) isolated L. pectinoschiza from pig cecal contents and feces using procedures developed to isolate "pectinophilic" bacteria from other environments. Pectinophiles are described as organisms able ferment only pectin and a few related compounds. Their approach was to isolate pectin-fermenting bacteria from a synthetic, salts-yeast-extract polygalacturonic acid medium and then to screen the isolates for the ability to use other carbohydrate sources. Although they were able to isolate L. pectinoschiza strains, these represented only a small percentage of the total obtained using this procedure.

IDENTIFICATION

Lachnospira multipara Lachnospira multipara is perhaps the only species of ruminal bacteria that can be presumptively identified on the basis of colonies formed in agar media (Bryant, 1963). Other species will on occasion form similar filamentous colonies, so identity must be confirmed by additional characterization. With nutrient-rich rumen fluid-carbohydrate media, surface colonies of *L. multipara* are large (3 to 5 mm in diameter), flat, white and filamentous (Bryant, 1974; Bryant, 1986b). Colonies within agar media are quite distinctive, appearing as white woolly balls. The typical appearance of *Lachnospira* is a curved rod (0.4 to 0.6 mm x 2 to 4 mm long) with bluntly rounded ends. In wet mount preparations, the organisms appear singly or in pairs of motile, curved rods. Most cells show some translational motility that is characterized by frequent tumbling and movement in circular

patterns. Although originally described as having polar, monotrichous flagellation (Bryant and Small, 1956b), it was later shown that the flagellum is subterminally or laterally attached. Cells generally stain weakly Gram-positive, but can be Gram-negative in older cultures. However, the ultrastructure of the cell wall appears to be that of a Gram-positive bacterium (Cheng et al., 1979; Hespell et al., 1993). Isolated peptidoglycans (PG) contained glucosamine, muramic acid, alanine, and meso-diaminopimelic acid. The PG preparations did not contain galactosamine, which was found in PG isolated from *Butyrivibrio fibrisolvens*.

Many *L. multipara* strains are capable of very rapid and extensive growth in carbohydrate-peptone-yeast extract media. Under these conditions, the cells often form very long chains or filaments (Bryant, 1986a). The cells in these structures are only slightly curved and have rounded ends. Quite often these structures become entwined with one another causing growth in liquid media to flocculate and readily settle out. This floc may be quite difficult to disperse and may adhere to glass surfaces. Growth of some strains can result in viscous cultures, suggesting the production of a polysaccharide material. An extracellular polysaccharide composed of rhamnose, mannose, galactose, and glucose has been isolated from cultures of the type strain (Mannarelli et al., 1990b).

Whereas all strains do not grow on cellulose or xylans and some strains may show weak growth on starch, all *L. multipara* strains rapidly ferment pectin and polygalacturonate (Bryant and Small, 1956b; Bryant, 1986; Bryant, 1963). The range of soluble carbohydrates used is limited and includes glucose, fructose, sucrose, cellobiose, esculin, salicin, and usually glucuronate. Xylose is weakly or variably fermented. The main fermentation products made from glucose are lactate, formate, acetate, ethanol, carbon dioxide, and some hydrogen gas. Small amounts of acetoin or propionate may be formed, but no butyrate or succinate is made. Biochemical tests for nitrate reduction, catalase, indole or hydrogen sulfide are negative. The GC of DNA from the type strain, D32^T is 38 mol% (buoyant density; Mannarelli et al., 1990b; T$_m$; Cotta, unpublished observation).

Lachnospira pectinoschiza The information available on characteristics of *Lachnospira pectinoschiza* is based on the description by Cornick et al., 1994. Surface colonies of *L. pectinoschiza* on agar media are opaque, circular with wavy edges, umbonate, and 3 to 5 mm in diameter. Growth only occurs under strictly anaerobic conditions. *L. pectinoschiza* cells are rod-shaped (0.36–0.56 mm wide × 2.4–3.1 mm long).

Although spore-like inclusions were sometimes observed in cells, survivors were not detected following heating or ethanol treatment. Cells are actively motile by means of peritrichous flagella (6–18 per cell). The cells stain Gram-positive and this cell ultrastructure was confirmed in electron micrographs of thin sections. The GC content of *L. pectinoschiza* strains ranged from 42–45 mol%. *L. pectinoschiza* grows rapidly on pectin and polygalacturonic acid, but few other carbohydrates are fermented. Lactose, gluconic acid, and cellobiose will also support growth. Fructose is used only after a long lag period. Growth occurs between 30–45°C, but not at 25°C or 50°C. The major products of pectin fermentation are acetic and formic acids, with smaller amounts of ethanol and carbon dioxide. Methanol is also produced during fermentation of pectin, presumably due to the action of pectin methylesterase. Ethanol is a major product when *L. pectinoschiza* is grown on lactose, cellobiose, and fructose. Lactate and hydrogen were detected. Tests for nitrate reduction, catalase, esculin and gelatin hydrolysis are negative.

CULTIVATION Members of *Lachnospira* are all strictly anaerobic bacteria, and appropriate steps should be employed to remove and exclude oxygen from growth medium (e.g., use of the technique of Hungate and modified by Bryant, 1972). The complex medium RGM (Table 2) can be used to grow strains of these bacteria as well as many other species of gastrointestinal anaerobes (Hespell et al., 1987). Peptides in the form of trypticase or other peptones are not required by *L. multipara*, but often stimulate growth when added to ammonia-containing defined media (Bryant and Robinson, 1962). Acetate was stimulatory to growth in defined media without peptone. *L. pectinoschiza* grows well in similar media with yeast extract (Cornick et al., 1994). The precise vitamin and other growth requirements for most strains of *Lachnospira* have not been determined. Strains are is generally grown at 37–39°C, but growth occurs between 30–45°C.

PRESERVATION Most strains of *Lachnospira* can be maintained for long times by storage of cultures in liquid nitrogen or in ultracold freezers (Hespell and Canale-Parola, 1970). Short-term storage (6–15 months) is possible by placing glycerol-containing cultures in normal (–20°C) freezers (Teather, 1982a). These organisms also can be preserved indefinitely by lyophilization.

PHYSIOLOGY Biochemical and physiological studies with *Lachnospira* sp. have mostly dealt with pectin degradation. *L. multipara* is capable of extensive maceration of clover and grass leaves, which can contain 2–8% pectin (Cheng et

al., 1979). *L. multipara* produces pectin lyase and pectin methylesterase activities, but appears to lack a polygalacturonase activity (Silley, 1985). An extracellular polygalacturonate lyase has been partially purified from strain 685 (Wojciechowicz et al., 1980). The enzyme required calcium for activity and was most active toward polygalacturonate, producing mainly the unsaturated digalacturonate from random attack on this substrate. Evidence for exopolygalacturonase activity in culture fluids of this strain (685) also was noted. In contrast, work with other strains (i.e., D15d and 2389) found no evidence for this last type of activity (Silley, 1985). However, both pectin lyase and pectin esterase activities were detected in these two strains. More recently, a detailed study of the kinetics of product formation by pectin lyase isolated from strain D15d indicated polygalacturonic acid was degraded by a random endolytic attack, producing primarily unsaturated trimers as the limit product (Preston et al., 1991). Production of dimer and trimer terminal products is consistent with the inability of *L. multipara* to ferment monomeric products of pectin degradation.

L. petinoschiza also produces pectin lyase and pectin methylesterase activities, and like *L. multipara* appears to lack an extracellular polygalacturonase activity (Cornick et al., 1994). Pectin lyase activity was more active against polygalacturonic acid, was calcium dependent, and was most active under alkaline conditions. Because unsaturated dimer was the only products detected, this enzyme may attack polygalacturonic acid in an exo-fashion.

ECOLOGY *Lachnospira multipara* is considered an ecologically important species of ruminal bacteria and can be a major constituent of the bacterial population under certain dietary conditions. *L. multipara* strains are the primary pectin fermenters isolated from ruminal contents of animals fed diets high in pectin (e.g., lush legumes, and citrus pulps). With animals fed rich ladino clover, 16–31% of the total bacterial isolates were *Lachnospira* strains (Bryant et al., 1960). Strains also have been isolated from ruminal contents of animals fed alfalfa hay when media containing pectin as the sole carbohydrate source are used (Dehority, 1969). In contrast to other pectin fermenting bacteria of the rumen (e.g., *Butyrivibrio fibrisolvens*), *L. multipara* strains have a limited range of carbohydrates that they ferment. Besides pectin, most strains ferment sucrose, fructose, and glucose, which are usually present at substantial levels in fresh, young forages. *L. multipara* is capable of invading and causing maceration of these types of plant materials (Cheng et al., 1979). During pectin fermentation, methanol is formed as a

product of pectin methyl esterase activity. In the rumen, this compound can be cross-fed to methanol-utilizing bacteria such as *Eubacterium limosum*. Evidence for this type of cross feeding has been demonstrated with cocultures of these species (Rode et al., 1981). Although *L. multipara* is very active in the degradation of pectin (a plant polysaccharide), synergisms in the growth and utilization of plant polysaccharides when cocultivated with other species of plant polysaccharide-utilizing species could not be demonstrated (Osborne and Dehority, 1989). The ecological significance of *L. pectinoschiza* in the swine gastrointestinal tract is unknown at this time.

Roseburia

INTRODUCTION The genus *Roseburia* contains a single species, *R. cecicola*, a Gram-negative, non-sporeforming, obligately anaerobic (slightly curved) rod-shaped bacterium isolated from the scrapings of cecal mucosa of mice. It is named after Theodor Rosebury, an American microbiologist who described and studied microorganisms indigenous to humans. The species name *cecicola* is used to indicate that this organism is a "cecum dweller" (Stanton and Savage, 1983a).

PHYLOGENY *Roseburia cecicola* is placed phylogenetically within cluster XIVa of the *Clostridium* subphylum, as defined by Collins et al., 1994. Cluster XIVa contains a diverse array of Gram-positive bacteria including members of the *Clostridium*, *Coprococcus*, *Eubacterium*, *Ruminococcus*, *Syntrophococcus*, *Lachnospira*, "*Acetitomaculum*" and *Butyrivibrio*. The phylogenetic relationship of *Roseburia*, as determined by 16S rRNA analysis (Cornick et al., 1994), indicates that *L. pectinoschiza* is the closest sequenced relative, with a similarity of 93%. (See Fig. 1.)

HABITAT *Roseburia cecicola* has been isolated from mucosal scrapings taken from the ceca of conventional laboratory mice (Stanton and Savage, 1983b), but the occurrence of this species in other animals is not known at this time. *R. cecicola* is motile, and motility and chemotaxis have been suggested as important characteristics for microorganisms that colonize mucosa and other tissues of animals. In mucosal scrapings, motile bacteria outnumber nonmotile bacteria by two to one (Stanton and Savage, 1983b). Stanton and Savage, 1984 showed that while motility was not essential for *R. cecicola* to colonize the ceca of gnotobiotic mice, it may be required for colonization of conventional mice. Using antibodies made to the flagellin protein of *R. cecicola*, this organism was shown to associate with the mucosal epithelium (Martin and Savage, 1984).
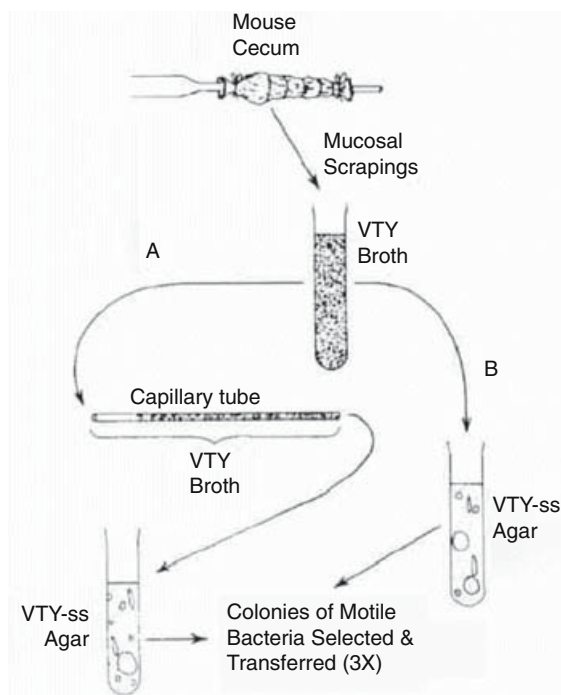
Fig. 3. Schematic representation of procedures to isolate motile bacteria from the mouse cecal mucosa. (A) Motile bacteria that swam through broth in a capillary tube were selected. (B) Motile bacteria were selected based on their colony morphology in semisolid medium. (by T. Stanton, reproduced with permission.)

Fig. 4. Phase-contrast micrographs of R. cecicola strain GM[T] cells (wet mount preparations). Cells were cultured in VTY broth and were in the exponential phase of growth. Each cell had a fascicle of 20–35 flagella (arrowheads). Bar = 5 μm. (by T. Stanton, reproduced with permission.)

ISOLATION *Roseburia cecicola* strain GM (type strain) was isolated using a procedure that selected for motile microorganisms present in the mucosal scrapings from mice ceca (Stanton and Savage, 1983b; Fig. 3). This approach involved inoculating a capillary tube filled with growth media. After incubation, the end of the tube opposite the inoculation end was broken off, some of the contents were removed and used to inoculate agar media to obtain individual colonies. This selection procedure is based on the concept that motile bacteria would migrate through the tube, whereas nonmotile bacteria would remain near the tube inoculation end. A selective medium containing soluble starch and the antibiotics nalidixic acid and tetracycline was useful in the selective enumeration of *R. cecicola* cells present in the cecum of mice inoculated with a defined population of microorganisms (penta-associated; Stanton and Savage, 1984). This medium, however, was not sufficiently selective to exclude all other cecal bacteria present in conventional mice.

IDENTIFICATION The information available on characteristics of *R. cecicola* is based on the description by Stanton and Savage, 1983a. Surface colonies of *R. cecicola* on agar media (0.7% Noble agar) are circular with a smooth edge, white, and 1.5 to 3.0 mm in diameter. The colonies have a granular appearance and are mucoid in texture. Subsurface colonies appear lenticular in shape, brownish white, and are about 1.0 mm in diameter. Growth only occurs under strictly anaerobic conditions. *R. cecicola* cells are curved rods (0.5 mm wide × 2.5 to 5.0 mm long) with rounded ends. Spores are not formed. Cells are actively motile and in wet mount preparations a single, large fascicle of flagella can be seen to arise from the concave cell side (Fig. 4). The fascicle is subterminally located and consists of 20 to 35 flagella. The cells stain Gram-negative and electron microscopic observations of thin sections indicate this bacterium has a multi-layered cell wall structure The presence of 2-keto-3-deoxyoctanoate (KDO) in cells is also indicative of a Gram-negative cell envelope (Martin and Savage, 1985). *R. cecicola* cells are catalase negative and the GC content of strain GM[T] (ATCC 33874) DNA is 42.3 mol%. *R. cecicola* does not ferment cellulose, xylan, or pectin, but does grow with starch as the energy source. Dextrin, amylopectin, and glycogen also support growth. Cellobiose, maltose, sucrose, raffinose, glucose, galactose, glucuronate, xylose, glycerol and sorbitol are fermented. During fermentation

of glucose, equimolar amounts of acetate are utilized and large amounts of butyrate and carbon dioxide are formed. Small amounts of ethanol also are made and hydrogen gas has been detected as a product. These characteristics clearly distinguish *R. cecicola* from other genera of Gram-negative, obligately anaerobic, non-sporeforming, rod-shaped bacteria (e.g., *Bacteroides*, *Butyrivibrio*, *Lachnospira*, *Selenomonas*, *Succinivibrio*).

CULTIVATION *Roseburia cecicola* is a strictly anaerobic bacterium and appropriate steps should be employed to remove and exclude oxygen from growth medium (e.g., use of the technique of Hungate and modified by Bryant, 1972). The complex medium RGM (Table 2) can be used to grow *R. cecicola* strains as well as many other species of gastrointestinal anaerobes (Hespell et al., 1987). The precise vitamin and other growth requirements for *Roseburia* strains have not been determined. *R. cecicola* grows optimally at 37°C and will also grow at 30°C, but not at 22°C or 45°C.

PRESERVATION *Roseburia cecicola* strains can be maintained for long times by storage of cultures in liquid nitrogen or in ultracold freezers (Hespell and Canale-Parola, 1970). Preservation by lyophilization under anaerobic conditions may be possible, and strains obtained from culture collections are provided in this form. Short-term storage (6–15 months) is possible by placing glycerol-containing cultures in normal (–20°C) freezers (Teather, 1982a).

PHYSIOLOGY Studies done with *R. cecicola* have centered on investigations of the role of motility in its colonization of the mouse gastrointestinal tract. Through the use of UV-induced motility mutants, Stanton and Savage, 1984 demonstrated that motility was not essential for the colonization of the cecum of germ-free mice. Motility, however, was required for successful colonization by *R. cecicola* when other species of gastrointestinal microorganisms were present. The flagella have been isolated from this organism using mechanical shearing (Martin and Savage, 1985) and are composed of a single protein (flagellin) with an estimated molecular weight of 42 kDa. The gene coding for this flagellin protein has been cloned and sequenced (Martin and Savage, 1988a). Based on the sequence data, the flagellin protein's molecular weight was estimated to be 31,370 Da, and comparisons with sequences of other bacterial flagellins revealed a greater similarity to those of Gram-positive rather than those from other Gram-negative organisms. During these studies, it was also dis-

covered that high molecular weight DNA could not be prepared from this organism if *R. cecicola* is exposed to oxygen prior to cell lysis (Martin and Savage, 1988b). A substance responsible for this activity was isolated from cell lysates. The low molecular weight (2.8 kDa) substance catalyzed the degradation of DNA by a mechanism consistent with the involvement of hydroxy radicals, which can be produced from other oxygen reduction products (αO_2^- and H_2O_2; O'Conner and Savage, 1993).

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CHAPTER 1.3.6

The Genus *Veillonella*

PAUL KOLENBRANDER

Introduction

Bacteria of the genus *Veillonella* Prévot are anaerobic, Gram-negative cocci (Rogosa, 1984). The type species was originally described by Veillon and Zuber (Veillon and Zuber, 1898) as *Staphylococcus parvulus* and renamed by Prévot (Prévot, 1933) as *Veillonella parvula*. Although all strains of *Veillonella* are phenotypically very similar, seven species are recognized by DNA homology analysis (Mays et al., 1982). The seven species can be identified by restriction fragment length polymorphism (RFLP) analysis of restriction enzyme digests of their 16S ribosomal DNA (Sato et al., 1997a). Veillonellae are found in the alimentary canal of warm-blooded animals and can constitute a major fraction of the bacteria on the epithelial surfaces of the human oral cavity.

Veillonellae are characterized by their unusual metabolism. They generally are unable to ferment carbohydrates including glucose but grow well anaerobically on lactate, pyruvate, malate or fumarate. They possess methyl malonyl-CoA decarboxylase, which belongs to a unique class of vectorial catalysts that convert the free energy of decarboxylation reactions into an electrochemical gradient of sodium ions (Dimroth, 1985). The enzyme catalyzes an essential reaction in the fermentation of lactate and provides an energy conservation mechanism, which contributes to their high cell yields per mole of lactate fermented.

Human oral veillonellae live in a lotic (flowing) environment and have developed mechanisms to colonize exposed surfaces. Veillonellae are only weakly adherent to hard and soft tissue surfaces, but they can adhere to other genera of oral bacteria (Gibbons and Nygaard, 1970; Hughes et al., 1988) and thus become part of multispecies bacterial networks (intergeneric coaggregations; Kolenbrander, 1988a). Many of these coaggregation partners are early colonizers that are capable of primary attachment to soft and hard tissue surfaces. Intergeneric coaggregation is highly specific in that veillonellae from one econiche, for example, the tongue dorsum, adhere to other bacteria found in that econiche but usually do not bind to bacteria from other econiches, for example, subgingival plaque (Hughes et al., 1988). Thus, interactions among oral bacteria appear to be important determinants in bacterial colonization of different surfaces.

Because of their unusual metabolic capabilities, including their use of the lactic acid produced by other oral bacteria from carbohydrate fermentation, the veillonellae form an essential link in the natural food chain. Their rise in numbers in the oral ecosystem immediately follows or parallels the proliferation of lactic acid-producing streptococci and actinomyces, both primary colonizers of the tooth surface and mucosal epithelium and both coaggregation partners of veillonellae. These two properties, intergeneric coaggregation and metabolic communication, may contribute significantly to the high numbers of veillonellae found in all dental plaque, whether obtained from healthy sites or diseased sites, which otherwise have very different bacterial populations (Dzink et al., 1988; L. V. H. Moore et al., 1987a; W. E. C. Moore et al., 1985).

Phylogeny

Although they are Gram negative by staining, the veillonellae are phylogenetically more closely related to Gram-positive species (Tanner et al., 1994; Willems and Collins, 1995). They belong in the *Sporomusa* subbranch of the *Clostridium* subphylum of the Gram-positive phylum of bacteria. The *Sporomusa* subbranch also includes closely related genera *Acidaminococcus* and *Megasphaera* as well as *Selenomonas*, which is related at a deeper level. Grouping the motile-rod selenomonads with the sessile-coccus veillonellae is consistent with the unusual shared presence of cadaverine and putrescine in their cell walls (Stackebrandt et al., 1985).

Taxonomy

The following are among the few positive phenotypic traits that are recognized for strains of veillonellae: small (0.3 to 0.5 μm), Gram-

negative cocci; usually occur in pairs; anaerobic growth on lactate or pyruvate, with accompanying production of propionic and acetic acids, CO_2, and H_2; and nitrate reduction (Rogosa, 1964a). Known variations of these properties include cells arranged in masses, short chains or single cells, and resistance to Gram-stain decolorization. Aerobic respiration of lactate or oxaloacetate occurs (Rogosa, 1964a) and some strains of V. *parvula* can grow aerobically in static culture on lactate (C. Hughes and P. Kolenbrander, unpublished observations) indicating that not all veillonellae are strict anaerobes. Some strains require putrescine or cadaverine (Rogosa and Bishop, 1964b) and some produce H_2S (Rogosa and Bishop, 1964c). Carbohydrates are not fermented (except for fructose by most strains of *V. criceti*, which are found in the oral cavity of hamsters; Mays et al., 1982).

Serological groupings were used to distinguish strains of animal and human origin (Rogosa, 1965a). Many of these strains were included in the 116 veillonellae examined for DNA homology (Mays et al., 1982). Seven DNA homology groups distinct at the species level were found (Table 1), and these correspond to the seven species recognized in {Bergey's Manual of Systematic Bacteriology} (Rogosa, 1984). Ribosomal RNA homology (16S and 23S) studies revealed three clusters with average intracluster homology values of greater than 90% and only 60 to 70% intercluster homology values (Johnson and Harich, 1983). *Veillonella atypica*, *V. caviae*, *V. dispar*, *V. rodentium*, and *V. parvula* belong to one cluster. *Veillonella criceti* and *V. ratti* are in a second cluster, and the third cluster consists of two strains of the "3312A" homology group, which comprise an unnamed species.

Veillonella are nonmotile and nonsporeforming. They do not use amino acids as a primary energy source and produce only sparse growth in a complex medium without added lactate, pyruvate, or other metabolizable intermediates. Together with *Acidaminococcus* and *Megasphaera*, they comprise the family *Veillonellaceae* (Rogosa, 1984). The GC content of the DNA of *Veillonella* species is 40.3 to 44.4 mol%, for

Acidaminococcus it is 56.6 mol%, and for *Megasphaera* it is 53.1 to 54.1 mol%. Unlike veillonellae, acidaminococci degrade amino acids, forming acetate and butyrate, while megasphaerae ferment both carbohydrates and organic acids but are inactive on amino acids (Rogosa, 1971). The end products of *Megasphaera* fermentation in a complex medium are acetate, butyrate, caproate, valerate, propionate, isobutyrate, and isovalerate. Colonies of *Veillonella* but not *Acidaminococcus* or *Megasphaera* exhibit a pink to red fluorescence on brain heart infusion agar when exposed to longwave (366 nm) UV light (Chow et al., 1975a). All species of *Veillonella* exhibit the fluorescence on brain heart infusion agar containing either sheep or horse blood (Brazier and Riley, 1988). The common human species, *V. atypica*, *V. dispar*, and *V. parvula*, do not fluoresce on blood-agar base containing either type of blood unless the agar contains δ-aminolevulinic acid. The fluorescence fades rapidly (5 to 10 minutes) on exposure to air and the pigment is typical of metal-free porphyrins.

Differentiation among the species of veillonellae on the basis of commonly used phenotypic tests is difficult, if not impossible. Decomposition of hydrogen peroxide, once used to differentiate between *V. parvula* and *V. alcalescens*, has been reported to vary among subcultures of the same strain (Mays et al., 1982). Thus, *V. alcalescens* has been renamed as *V. parvula*. By using defined culture conditions and cell-extraction procedures, distinct polyacrylamide gel electrophoretic patterns of soluble proteins were found for each species (Mays et al., 1982).

Habitat

Normal Human Oral Flora

Veillonellae are the most numerous anaerobes in human saliva (Sutter, 1984), where they are found at concentrations of 1.7 to 6.9 x 10^7/ml (Richardson and Jones, 1958; Rogosa et al., 1958), and constitute from 5% (Liljemark and Gibbons, 1971) to about 16% (Hardie and Bowden, 1974) of the cultivable anaerobic flora. Almost 10% of the total cultivable anaerobic flora from the tongue dorsum consist of veillonellae (Liljemark and Gibbons, 1971). In supragingival plaque, estimates of veillonellae as a percentage of total bacteria have ranged from about 1% (Liljemark and Gibbons, 1971) to 5% (L. V. H. Moore et al., 1987a; W. E. C. Moore et al., 1985). Direct comparison of the subgingival and supragingival plaque bacteria isolated from healthy sites indicates that about 4 and 5%, respectively, are veillonellae (L. V. H. Moore

Table 1. Characteristics of the type strains of the seven recognized species of the genus *Veillonella*.

Species	ATCC no.	Serogroup	Origin
V. atypica	17744	V	Human
V. caviae	33540	none	Guinea pig
V. criceti	17747	I	Hamster
V. dispar	17748	VII	Human
V. parvula	10790	VI	Human
V. ratti	17746	III	Rat
V. rodentium	17743	II	Hamster

et al., 1987b; W. E. C. Moore et al., 1985). While the veillonellae found on the tongue dorsum are either strains of *V. atypica* or *V. dispar* (Hughes et al., 1988), those found in subgingival plaque samples are primarily *V. parvula* (L. V. H. Moore et al., 1987a; W. E. C. Moore et al., 1985). Veillonellae isolated from saliva and buccal mucosa are mostly *V. atypica* and *V. dispar* (Hughes et al., 1988). *V. atypica* is one of three species of oral bacteria that contribute most to changes in subgingival flora composition during puberty and is one of two species that is particularly associated with teenagers (W. E. C. Moore et al., 1993). *V. parvula* is isolated from subgingival plaque with *Actinomyces odontolyticus*, and these two bacterial species are among the most commonly detected anaerobes in subcutaneous abscesses of intravenous drug users (Summanen et al., 1995). Of the seven taxonomically recognized *Veillonella* species (Rogosa, 1984), *V. atypica*, *V. dispar*, and *V. parvula* are indigenous to the respiratory and intestinal tracts of humans, and the other species are distributed among ruminants, rodents, and pigs.

Experimental Animal Models

Veillonellae are among the predominant microbes of the saliva, tongue dorsum, buccal mucosa, and gingival crevice of both the plaque-susceptible and plaque-resistant rats that were derived from the Wistar Kyoto strain (Isogai et al., 1985). On the basis of total anaerobic cultivable flora, veillonellae were the most numerous bacteria from subgingival plaque of both STR/N periodontitis-susceptible mice and Swiss-Webster periodontitis-nonsusceptible mice (Wolff et al., 1985). Their numbers remained constant from three to thirteen months, the time interval during which other microbial changes occurred with the advancing periodontitis in the STR/N mice. Thus, the number and distribution of veillonellae in these experimental animals are very similar to those observed in the human oral cavity.

Isolation

The following selective medium was designed by (Rogosa, 1956) and modified (Rogosa et al., 1958) and has been used to isolate veillonellae from the human oral cavity (Hughes et al., 1988).

Distilled water is added to a final volume of 1 liter and the pH is adjusted to 7.5 before autoclaving. Vancomycin is added at 7.5 μg per ml before pouring plates. The plates are incubated anaerobically under an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 at 37°C for 48 hours. A commercial medium based on this recipe is

Table 2. Selective Medium for *Veillonella* Species

Tryptone	5.0 g
Yeast extract	3.0 g
Sodium thioglycollate	0.75 g
Basic fuchsin	0.002 g
Tween 80	1.0 g
Sodium lactate (60% w/w)	21.0 ml
Agar	15.0 g
$K_2HPO_4 \cdot 3H_2O$	3.0 g
KH_2PO_4	1.0 g
$CaCl_2 \cdot 2H_2O$	0.15 g
NH_4Cl	0.5 g
$MgCl_2 \cdot 6H_2O$	0.05 g
$Fe_2(SO_4)_3$	0.05 g
Yeast extract	0.1 g
Trypticase peptone	0.1 g
Resazurin	0.001 g
$Na_2S \cdot 9H_2O$	0.5 g
Sodium lactate (50%)	10 ml
$NiSO_4 \cdot 6H_2O$	0.5
$MnCl_2 \cdot 4H_2O$	0.5
$FeSO_4 \cdot 7H_2O$	0.5
$ZnSO_4 \cdot 7H_2O$	0.1
H_3BO_3	0.1
$Na_2MoO_4 \cdot 2H_2O$	0.05
$CoCl_2 \cdot 2H_2O$	0.05
$CuSO_4 \cdot 5H_2O$	0.005

available (Bacto *Veillonella* Agar, Difco Laboratories, Detroit, Michigan).

A medium used to enrich for an isolate identified as *Veillonella* spp. from a methanogenic upflow anaerobic sludge blanket reactor is as follows (Slobodkin and Verstraete, 1993):

$KH_2PO_4 \cdot 3H_2O$	3.0 g
KH_2PO_4	1.0 g
$CaCl_2 \cdot 2H_2O$	0.15 g
NH_4Cl	0.5 g
$MgCl_2 \cdot 6H_2O$	0.05 g
$Fe_2(SO_4)_3$	0.05 g
Yeast extract	0.1 g
Trypticase peptone	0.1 g
Resazurin	0.001 g
$Na_2S \cdot 9H_2O$	0.5 g
Sodium lactate (50%)	10 ml

Distilled water is added to a final volume of 1 liter. The medium also contains 1.0 ml of trace elements solution per liter of the following composition (in g/liter of distilled water):

$NiSO_4 \cdot 6H_2O$	0.5
$MnCl_2 \cdot 4H_2O$	0.5
$FeSO_4 \cdot 7H_2O$	0.5
$ZnSO_4 \cdot 7H_2O$	0.1
H_3BO_3	0.1
$Na_2MoO_4 \cdot 2H_2O$	0.05
$CoCl_2 \cdot 2H_2O$	0.05
$CuSO_4 \cdot 5H_2O$	0.005

Ten-ml aliquots of medium were anaerobically dispensed in 17-ml capacity test tubes, gassed with $N_2:CO_2:H_2$ (84:8:8, v/v), and sealed with butyl rubber stoppers and screw caps. Heat sterilization (121°C for 15min) of the

medium caused the formation of an insoluble black precipitate, which appeared to function as a biofilm support for the veillonellae (Slobodkin and Verstraete, 1993).

A defined medium supporting growth of all seven species of *Veillonella* has been tested (Durant et al., 1997). No growth was observed when lactate was omitted. Aspartate, threonine, arginine, and serine when added to the defined medium were metabolized to acetate and propionate.

Nonselective media that have been successfully used to determine the bacterial flora (including veillonellae) of healthy subgingival sites or of periodontally diseased sites are: Brucella agar supplemented with sheep blood and menadione (Williams et al., 1976), MM10 medium (Slots, 1977), trypticase soy blood agar (Newman et al., 1978), and supplemented brain heart infusion blood agar (W. E. C. Moore et al., 1982a).

Identification

Thin sections of veillonellae viewed by electron microscopy exhibit typical Gram-negative surface layers consisting of an outer membrane, peptidoglycan layer, and cytoplasmic membrane (Bladen et al., 1967; Bladen and Mergenhagen, 1964; Tortosa et al., 1981). Phase contrast photomicrographs of the three human species of *Veillonella* are shown in Figure 1. The cells are spherical and are either single or in short chains of two to four cells. Most of the cells are 0.3 to 0.5 μm in diameter.

Identifying fresh isolates to the genus level of *Veillonella* is relatively straightforward. Gram-negative, small spherical cells that reduce nitrate and grow on lactate or pyruvate but not on glucose under anaerobic atmosphere are the primary characteristics. Gas chromatographic analysis of the fermentation end products indicates acetic and propionic acids are the major products, and H_2 and CO_2 are generated as well (Holdeman et al., 1977). Pyrolysis gas chromatography of lipopolysaccharides has been reported to be useful in differentiating *Veillonella*, *Fusobacterium*, and *Bacteroides* (Dahlén and Ericsson, 1983).

Classification to the species level by conventional phenotypic and biochemical tests relies on numerous negative reactions and cannot distinguish the seven species without labor-intensive methods. Methods involving analyses of cellular fatty acids have been developed (L. V. H. Moore et al., 1987b) and found useful in distinguishing the three human species, *V. atypica*, *V. dispar*, and *V. parvula* (Hughes et al., 1988). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of veillonellae proteins yields distinct profiles for each of the seven species (Mays et al., 1982; Sato et al., 1997b; Tanner et al., 1992).

Rapid-identification systems are being developed, such as RapID ANA II (Innovative Diagnostic Systems, Inc., Atlanta, GA), ANI Card (Vitek Systems, Hazelwood, MO), and AN-Ident and API ZYM (Analytab, Plainview, NY). Another system, the BBL Crystal Anaerobe Identification System, correctly identified the two *V. parvula* strains included in the panel of
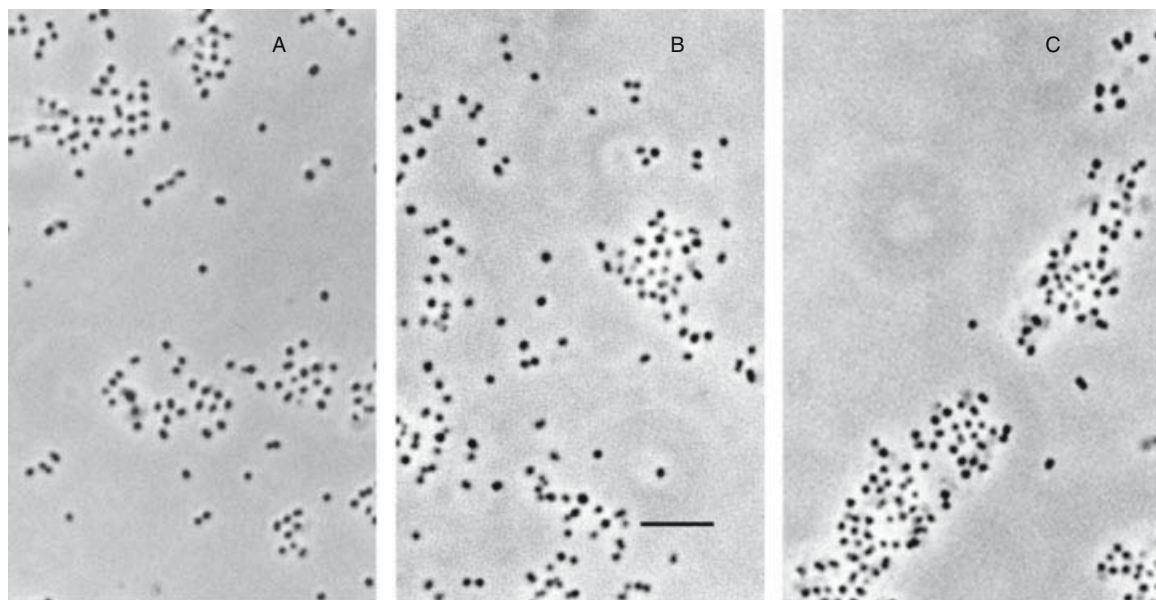
Fig. 1. Phase contrast photomicrographs of (A) *Veillonella atypica* PK1910, (B) *Veillonella dispar* PK1913, and (C) *Veillonella parvula* PK1914 isolates from the human oral cavity. Bar = 10 μm. All figures are at the same magnification.

322 clinically significant anaerobes (Cavallaro et al., 1997).

Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S ribosomal DNA (16S rDNA) can rapidly and reliably distinguish the seven species of *Veillonella* (Sato et al., 1997a, b). The 16S rRNA genes are amplified by PCR using the following primers (forward primer sequence: 5′-GTG CTG CAG AGA GTT TGA TCC TGG CTC AG-3′; reverse primer sequence: 5′-CAC GGA TCC TAC GGG TAC CTT GTT ACG ACT T-3′). Digestion of the PCR products of the 16S rDNA using the restriction endonuclease MnlI yields six RFLP patterns for the seven type strains of *Veillonella* (Sato et al., 1997b). The RFLP patterns of *V. atypica* and *V. caviae* are the same, but they can be distinguished by digestion of the 16S rDNA with Sau3AI.

Preservation

Strains survive freezing at −80°C, preferably suspended in blood, and lyophilization.

Physiology

The unusual physiology and special energy-conservation mechanism of veillonellae have been reviewed (Delwiche et al., 1985; Dimroth, 1985; Dimroth, 1994). The veillonellae are unable to use glucose to support growth, and they do not have a functional hexokinase (Rogosa et al., 1965b). Radiolabeled glucose is not incorporated into bacterial cell compounds (Winter and Delwiche, 1975), suggesting that the glucose phosphotransferase system is also absent. Ribose and fructose can be incorporated into nucleic acid (Kafkewitz and Delwiche, 1972) or lipopolysaccharide (Tortorello and Delwiche, 1983; Winter and Delwiche, 1975) but are not fermented. Veillonellae can utilize lactate as carbon and energy source for growth. Pyruvate, fumarate, malate, α-ketoglutarate, and some purines also are substrates for growth. The major end product of fermentative growth is propionic acid along with acetic acid, carbon dioxide and hydrogen. Propionic acid but not acetic acid is toxic to cultured human gingival fibroblasts and may play a role in host tissue damage (Singer and Buckner, 1981).

Fermentation of lactate proceeds through four-carbon dicarboxylic acid intermediates and involves a novel energy conversion mechanism of biotin-dependent sodium-transport methylmalonyl-CoA decarboxylase (Hilpert and Dimroth, 1983). The decarboxylase is membrane bound, is specifically activated by Na$^+$ ions, and

converts part of the energy of the highly exergonic decarboxylation reaction into a sodium ion gradient. This electrochemical gradient of Na$^+$ ions may be used to drive the active transport of the growth substrate. This methylmalonyl-CoA-decarboxylase, which is found in both veillonellae and *Selenomonas ruminantium* (Melville et al., 1988), the oxaloacetate decarboxylase from *Klebsiella pneumoniae* (formerly *K. aerogenes*; Dimroth, 1981), and the glutaconyl-CoA decarboxylase from *Acidaminococcus fermentans* (Buckel and Semmler, 1982) and *Fusobacterium nucleatum* (Beatrix et al., 1990) have been found only in anaerobic bacteria, where they are induced by their respective fermentation pathway substrates (e.g., lactate with the veillonellae). The physiological significance of these enzymes is two-fold. They are essential for growth on appropriate substrates, and they offer a mechanism for energy conservation and thus provide additional energy to that obtained from the fermentation itself.

The five subunits of the *m*ethyl*m*alonyl-CoA *d*ecarboxylase from *V. parvula* are encoded by a clustered set of genes *mmdA* (α), *mmdD* (δ), *mmdE* (ε), *mmdC* (γ), and *mmdB* (β) (Huder and Dimroth, 1993). The α–subunit is 60% identical to the carboxyltransferase domain of rat liver propionyl-CoA carboxylase. The β–subunits of methylmalonyl-CoA decarboxylase and oxaloacetate decarboxylase of *V. parvula* and *K. pneumoniae*, respectively, are 61% identical. Regarding the mechanism of Na$^+$ transport, much of the identical sequence is in the hydrophobic region, and it contains two conserved aspartic acid residues within the putative membrane-spanning helixes (Huder and Dimroth, 1993). Biotin binds to the γ–subunit, which is 29 to 39% identical with biotin domains of other biotin enzymes. The small ε–subunit (5888-Da) is 47% identical to the C-terminal region of the δ–subunit. The five *mmd* genes were cloned and expressed in Escherichia coli to analyze the role of the δ– and ε–subunits (Huder and Dimroth, 1995). The δ–subunit appears to be involved in assembly of the enzyme complex and, thus, necessary for catalytic function; the ε–subunit has no catalytic function but may increase the structural stability of the five-subunit methylmalonyl-CoA decarboxylase complex (Huder and Dimroth, 1995).

V. parvula cannot grow on succinate as sole carbon and energy source, but can decarboxylate succinate during fermentation of lactate or malate (Denger and Schink, 1992; Janssen, 1992). Addition of succinate increases the growth yields by 2.4 to 3.5 g cell dry mass per mole of succinate, which demonstrates that energy is conserved through succinate decarboxylation. This yield reflects a small free energy

change from decarboxylation and is equivalent to only a third of an ATP, indicating that it helps to save energy but is not sufficient to support growth by itself. *V. parvula* uses pyruvate carboxylase and methylmalonyl-CoA decarboxylase, rather than a transcarboxylase as used by *Propionibacterium* spp., for formation and decarboxylation of C$_4$-dicarboxylic acids (Denger and Schink, 1992).

Gluconeogenesis occurs by a reversed glycolytic sequence to glucose-6-phosphate from lactate (Rogosa et al., 1965b), or by an alternate pathway through malate and glyoxylate to 3-phosphoglycerate (Pestka and Delwiche, 1983), rather than going directly from lactate through 2-phosphoglycerate to 3-phosphoglycerate. Lactate carbon is incorporated into six-carbon sugars (Ng and Hamilton, 1974).

The peptidoglycan of some veillonellae contains putrescine or cadaverine, which are required for normal growth. Putrescine and cadaverine are covalently linked to the α-carboxyl group of the D-glutamic acid residue; both are equally incorporated into peptidoglycan, and during normal growth, they occupy over 40% of the residues (Kamio, 1987a; Kamio and Nakamura, 1987b). Other normal constituents of the peptidoglycan are *N*-acetylglucosamine, *N*-acetylmuramic acid, L-alanine, D-glutamic acid, meso-diaminopimelic acid, and D-alanine. The lipopolysaccharide composition includes glucosamine, tridecanoic acid, and 3-hydroxytridecanoic acid in the lipid moiety and contains 2-keto-3-deoxyoctonic acid, glyceromannoheptose and variable components including galactose, rhamnose, glucosamine, and glucose in the polysaccharide moiety (Hewett et al., 1971; Hofstad and Kristoffersen, 1970). The lipopolysaccharide has been shown to be an endotoxin (Mergenhagen et al., 1961). Endotoxin from both *V. parvula* and *V. atypica* suppress microbicidal activity of human neutrophils but do not affect monocyte function (Foca et al., 1990).

The cytoplasmic membrane of *V. parvula* contains an unusual class of polar lipids called plasmalogens (Olsen, 1997), which is found among anaerobes and certain groups of protozoa and most tissues of higher eukaryotes. The plasmalogens are ether lipids and represent chemically the condensation of a fatty aldehyde to glycerol resulting in a vinyl ether. Plasmalogens, plasmenylethanolamine and plasmenylserine, are present in *V. parvula* and are thought to have specific roles in membrane organization and stability (Olsen, 1997).

Purines may be fermented with the production of acetate, propionate, ammonia, urea, CO$_2$, and H$_2$ (Whiteley and Douglas, 1951). Large amounts of gas may be formed. Nitrate is reduced through nitrite and hydroxylamine to ammonia, which is assimilated (Inderlied and Delwiche, 1973; Ruoff and Delwiche, 1977; Yordy and Delwiche, 1979). An unusual type of xanthine dehydrogenase has been purified and characterized from *V. atypica* (Gremer and Meyer, 1996). It is a129-k Da molybdenum-containing iron-sulfur flavoprotein composed of a noncovalent heterotrimer with 82.4-kDa, 28.5-kDa and 18.4-kDa subunits. *V. parvula* and other rumen bacteria produce dipeptidyl peptidases, which cleave dipeptides from the amino terminus of longer peptides. This contributes to the inefficient use of dietary nitrogen by the host animal and causes waste of nitrogenous feed resources and environmental pollution (Wallace et al., 1997).

Genetics

No genetic system has been reported for any of the veillonellae.

Ecology

Ecological Succession Before and After Tooth Eruption

Shortly after birth, veillonellae appear in the human mouth. They are infrequently detectable in newborns one to eight days old but are found in 75% of infants that are 101 days old and in 100% of one-year-old children (McCarthy et al., 1965). The increase in numbers of veillonellae occurs following the colonization of the predominant oral bacterial species, *Streptococcus salivarius*. In a group of 30 edentulous infants ranging in age from 1 to 7 months, *Veillonella* spp. along with *Fusobacterium nucleatum* and *Prevotella melaninogenica* were the predominant anaerobes (Könönen et al., 1992) found in about 70% of the samples of mucosal surfaces and saliva. These children were tested at 32 months (mean age) with primary dentition; *Veillonella* spp. occurred at the same level as in infants, but the other two species were found in every child (Könönen et al., 1994). Bacteria obtained from the tongues of children of ages 8 to 13 months are diverse in composition (Milnes et al., 1993). While *S. salivarius* is the predominant organism and isolated from 94% of the samples, *Veillonella* spp. are isolated from 73% of the samples, and they were consistently present over the 16 month-duration of the study.

Removal of teeth also causes a change in microbial composition, but veillonellae remain the dominant anaerobic organism in the saliva of geriatric edentulous persons (Sato et al., 1993). Veillonellae are not known to adhere well to buccal epithelial cells (Liljemark and Gibbons,

1971), whereas *S. salivarius* adheres very well (Gibbons and van Houte, 1971). Some veillonellae coaggregate with *S. salivarius*, which may provide a mechanism for veillonellae to adhere in the oral cavity (Hughes et al., 1988).

Ecological Succession from Healthy Gingiva to Periodontal Disease

V. parvula is the predominant *Veillonella* in human dental plaque and constitutes between 93 and 98% of the cultivable veillonellae in healthy subgingival sites (W. E. C. Moore et al., 1985), and its numbers increase in gingivitis as compared with healthy subgingival sites (L. V. H. Moore et al., 1987a; Slots et al., 1978). *V. parvula* is a member of the microbial consortium consistently associated with successful osseointegrated dental implants (Lee et al., 1999). Although *V. atypica* and *V. dispar* are usually present in plaque, they normally occur in low numbers and are not associated with any oral diseases.

Two early studies were conducted to examine shifts in the bacterial population of cleaned hard surfaces in the adult human oral cavity. In the first investigation (Slack and Bowden, 1965), an artificial device was positioned to simulate an interproximal site, and in the other study (Ritz, 1967), a professionally cleaned tooth surface was examined. Both studies indicated that veillonellae were detectable along with streptococci after only 24 hours, and that after 3 days, fusobacteria, actinomyces, and a few other bacterial types also were present. Even when the plaque composition on a cleaned tooth surface was monitored at minute or hourly intervals for the first day, veillonellae were rarely found in samples during the first 24 hours (Socransky et al., 1977; Theilade et al., 1982) but constituted about 20% of the strains isolated from 1- and 3-day old dental plaque (Theilade et al., 1982).

Other surveys indicate that veillonellae remain a significant part of the population in both healthy and diseased sites (Dzink et al., 1985; Loesche and Syed, 1978; Syed and Loesche, 1978) and are among the 10 most numerous species of subgingival plaque bacteria under all conditions examined (Dzink et al., 1988; L. V. H. Moore et al., 1987b; W. E. C. Moore et al., 1985), including experimental gingivitis (W. E. C. Moore et al., 1982b), localized juvenile periodontitis (W. E. C. Moore et al., 1985; Williams et al., 1985), adult chronic moderate periodontitis (W. E. C. Moore et al., 1983), and rapidly progressing generalized periodontitis (W. E. C. Moore et al., 1982). In all of these extensive investigations of the microbial composition of dental plaque, veillonellae are always found as numerically significant members of a consortium of bacterial species. Streptococci and actinomyces repopulate cleaned tooth surfaces, and both of these organisms are coaggregation partners of *V. parvula* (Hughes et al., 1988). Further, populations in periodontally diseased sites are dominated by fusobacteria, which are coaggregation partners of all three human *Veillonella* species (Kolenbrander et al., 1989).

Metabolic Communication

Digestion of proteins by protease-secreting bacterial inhabitants of anaerobic consortia would provide amino acids such as L-serine, which is known to enhance lactate metabolism of veillonellae (Hoshino, 1987). Menaquinones, a potential source of vitamin K for other bacteria, are present in some veillonellae (Ramotar et al., 1984). Mixtures of *V. parvula* and *Haemophilus parainfluenzae*, both isolated from the gastric juice of achlorhydric patients, accumulated nitrite during nitrate reduction, and the nitrite concentration could be decreased by including the nitrite-reducing *Neisseria subflava* or *Streptococcus sanguis* (Forsythe and Cole, 1987). Coculturing amylolytic rumen bacteria and lactate-utilizing bacteria (veillonellae) on starch usually produced higher growth yields (Marounek and Bartos, 1987). Growth of *Veillonella* with the lactate-producing *Streptococcus mutans* (Distler and Kroncke, 1980) or lactate- and succinate-producing *Actinomyces* (Distler and Kroncke, 1981) or with *Eubacterium saburreum* (Mashimo et al., 1981) has been demonstrated.

A two-species chemostat consisting of an aerobic bacterium, *Comamonas testosteroni*, and anaerobic *V. parvula* was established with dual limitation of L-lactate and oxygen (Gerritse et al., 1992). Although catalase is detectable in cultures of *V. parvula* and cultures can grow in the presence of oxygen at concentrations below $0.8\,\mu M$ (de Vries et al., 1978), results of this study showed that superoxide anion radicals and catalase were produced. Inhibition of growth of *V. parvula* in air was correlated with a rapid but reversible inactivation of lactate dehydrogenase (de Vries et al., 1978). *C. testosteroni* is an obligate aerobe that can grow on L-lactate as a sole carbon and energy source and can use propionate and acetate, the two major fermentation products of *V. parvula* (Gerritse et al., 1992). Coexistence of the two species occurred only when the chemostat culture was oxygen limited. *C. testosteroni* grew primarily on the end products of lactate fermentation by *V. parvula*, and apparently protected the veillonellae from oxygen inhibition by reducing the oxygen concentration to a tolerable level for the veillonellae (Gerritse et al., 1992). The veillonellae were better competitors for L-lactate even under low oxygen concentrations, which may explain why

the biomass of the veillonellae varied little, but the biomass of *C. testosteroni* increased linearly with increasing oxygen supply. Thus, it appears that the most important determinant for coexistence is the affinity of *C. testosteroni* for oxygen and the sensitivity of *V. parvula* for oxygen.

A consortium of nine oral bacteria in a glucose-limited chemostat was monitored for changes in the composition of the bacterial population accompanying pH changes (McDermid et al., 1986). At pH 7.0 veillonellae and streptococci were most numerous, and, at pH 4.1, veillonellae were numerically second only to *Lactobacillus rhamnosus* (formerly *L. casei*). They regained their same predominance with the streptococci when the pH was returned to 7.0. When the consortium was subjected to a pulse of glucose rather than to a continuous supply, *V. dispar* was the most numerous organism following all the glucose pulses, and it was especially dominant at low pH values of 5.5 to 4.5 (Bradshaw and Marsh, 1998). These experiments show that veillonellae are able to effectively utilize lactic acid produced by a variety of oral bacteria including streptococci and lactobacilli (Delwiche et al., 1985; Mikx and van der Hoeven, 1975) in either acidic or neutral pH environments. In a separate study, persistence and eventual predominance of *V. dispar* along with streptococci in a glucose-limited chemostat inoculated with a tongue scraping are attributed to the ability of veillonellae to utilize the lactic acid end products of other oral bacteria (Basson and van Wyk, 1996).

Similar metabolic communications in vivo are suggested by the observation that incipient carious lesions in the teeth of children have an increased number of *S. mutans* and *Lactobacillus* spp. along with increased numbers of *Veillonella* spp. (Boyar and Bowden, 1985). Also, veillonellae are associated with the dense infections of *S. mutans* on susceptible surfaces related to developing lesions of nursing caries in young children (Milnes and Bowden, 1985). The highest proportions of *Veillonella* spp. were found at sites with the highest numbers of mutans streptococci (Babaahmady et al., 1997). In contrast, veillonellae may protect the tooth surface by removal of the stronger lactic acid and replacement with its own metabolic end products, the weaker acetic and propionic acids. To function in this protective role, veillonellae should increase their numbers quickly in response to small amounts of lactic acid. In accord with this hypothesis, the predominant supragingival flora of caries-free Tanzanian children consisted of unusually high proportions of *Veillonella* spp. (30% to 40% of the total cultivable microflora) along with the oral streptococci (Kilian et al., 1979). Plaque from caries-free adults also contains higher levels of

Veillonella species than does plaque from caries-susceptible adults (Minah et al., 1981).

With gnotobiotic animals, it was shown that *S. sanguis* or *S. mutans* was more cariogenic when either species mono-infected an animal than when associated with veillonellae (Mikx et al., 1972). This caries reduction was interpreted to be due to conversion of streptococcal-produced lactic acid to the weaker acids, acetic and propionic acids, by the veillonellae. A reduction in lactic acid and an increase in acetic acid was shown to occur in dental plaque of gnotobiotic rats hosting an experimental symbiosis of veillonellae and cariogenic streptococci (van der Hoeven et al., 1978). Compared to the high numbers of *S. mutans* found in nursing caries of children, the number of *S. mutans* required to cause caries in gnotobiotic animals is quite low. The reduction in caries by association with veillonellae may occur through the rapid growth response by veillonellae to small amounts of lactic acid, while the dense streptococcal population in nursing caries maintains a high lactic acid concentration and little significant reduction in caries occurs even in the presence of veillonellae.

Epidemiology

Veillonellae are frequently isolated from the inferior conjunctival sacs of normal eyes and anophthalmic cavities (Campos et al., 1994), and they are commonly found in extraoral sites in patients undergoing medical procedures or patients with infections. *V. parvula* is one of the three most frequently isolated anaerobic organisms in ventilator-associated pneumonia (Doré et al., 1996), and it was the only anaerobe isolated in a second study of ventilator-associated pneumonia, where it was considered a nonpathogen (Marik and Careau, 1999). It is isolated as one of several species from ultrasound-guided percutaneous transthoracic aspiration of patients with obstructive pneumonitis (Liaw et al., 1994) and lung abscesses (Mori et al., 1993). It is part of the increased oral anaerobic bacterial load isolated from biofilms associated with human oral carcinomas (Nagy et al., 1998).

Veillonellae are much less frequently isolated from infection sites than the anaerobic streptococci (*Streptococcus*, *Peptococcus*, *Peptostreptococcus*) but are frequently found in neck space infections of children (Ungkanont et al., 1995). They have been isolated as the only bacterial type from patients with endocarditis (Greaves and Kaiser, 1984; Loewe et al., 1946), hepatic abscesses (Lambe et al., 1974), and pleuropulmonary infections (Bartlett and Finegold, 1972). They were present in 14 of 62 specimens of chronic sinusitis (Heineman and Braude, 1963)

and were the only genus isolated in some cases (Frederick and Braude, 1974). They also have been found in bite-wound infections (Goldstein et al., 1984), gynecological infections (Chow et al., 1975a), intra-abdominal abscesses (W. E. C. Moore et al., 1969), pelvic abscesses (Williams, 1977), septicemia and osteomyelitis (Barnhart et al., 1983; Borchardt et al., 1977), pleuropulmonary infections (Bartlett et al., 1974; Martin, 1974), and periodontal disease (W. E. C. Moore, 1987). When isolated from blood cultures, they usually are mixed with other species and probably occur mixed with other species in most isolations from most sources.

They are like the other anaerobes in their susceptibility to the various antimicrobials, and many are resistant to tetracycline at concentrations greater than 10 μg/ml. Thirteen strains of *Veillonella* were among the 601 clinical isolates of anaerobic bacteria tested for minimal inhibitory concentrations of 10 antimicrobial agents (Martin et al., 1972). In periodontal patients undergoing tetracycline therapy, *Veillonella* species along with *Streptococcus* and *Neisseria* that are resistant to tetracycline with minimal inhibitory concentrations as high as 128 μg/ml are frequently isolated (Williams et al., 1979). The DNA from these species, as well as from tetracycline-resistant *Fusobacterium nucleatum* and *Peptostreptococcus anaerobius*, hybridize with a TetM probe, prepared from one of the streptococcal tetracycline-resistance determinants (Roberts and Moncla, 1988). Another tetracycline-resistant determinant normally associated with Gram-positive bacteria, TetL, has been detected in *V. parvula* (Pang et al., 1994). These results suggest that these determinants can exist and confer tetracycline resistance in either aerobic or anaerobic Gram-negative and Gram-positive bacteria.

Veillonellae are readily isolated from the oral cavity of patients, but the numbers isolated vary depending on the patient's oral and medical condition. As compared to the normal flora of the oral cavity, veillonellae in patients with severe xerostomia due to Sjögren's syndrome are greatly reduced in number, whereas the numbers of *Candida* spp. and of *Staphylococcus aureus* increase (MacFarlane, 1984). Veillonellae are isolated as part of the microbial flora from root canal specimens and may be important in endodontic infections (Burnett and Schuster, 1978). *V. dispar* is found in much higher numbers and greater frequency than *V. parvula* in subgingival plaque obtained from patients with non-insulin-dependent diabetes mellitus (Zambon et al., 1988). Despite the variation in numbers of veillonellae with disease condition, these anaerobes appear not to be associated with the risk of oral disease. In a study of dental decay of children's molars shortly after eruption, *S. mutans* and lactobacilli are associated with the development of decay, whereas veillonellae, although consistently present, are not (Loesche et al., 1984). Veillonellae are found in nearly all samples from older adults with a high root surface caries risk, but are not correlated with the disease (Ellen et al., 1985). Thus, the occurrence of veillonellae in human oral infection may be a consequence of ecological changes in the infection site rather than a direct pathogenic property of these bacteria.

Disease

Veillonellae are ubiquitous in the oral cavity, but they produce disease only in rare instances. Two fatal cases of *Veillonella* bacteremia have been reported where *V. parvula* was isolated as a pure culture from the patient's blood (Liu et al., 1998). Veillonellae have been implicated as pathogens in several body sites including bone, heart, liver, lungs, sinuses and the central nervous system. Veillonellae cause bacteremia (Fisher and Denison, 1996) and were found as a monospecies infection in infectious myositis in an immunocompromised patient (Beumont et al., 1995), prosthetic valve endocarditis (Houston et al., 1997; Loughrey and Chew, 1990; Zussa et al., 1994), spinal osteomyelitis (Singh and Yu, 1992), septic sacroiliitis (Pouchot et al., 1992), cervicofacial infection (Jones et al., 1989), or part of multispecies infections in skin and soft tissue of intravenous drug users (Summanen et al., 1995; Wexler et al., 1998). A brief review summarizing the *Veillonella* infections in children cites the recovery of *Veillonella* spp. in 4% of specimens submitted for anaerobic culturing (Brook, 1996). Most of the infections were multispecies but four specimens had pure cultures of veillonellae. *Veillonella* spp. are part of a mixed species population in the upper gut in patients with small intestinal bacterial overgrowth syndrome, where the proximal part of the small bowel harbors abnormally high bacterial numbers (greater than 10^5 bacteria per ml of intestinal juice) for a long time (Bouhnik et al., 1999).

Applications

The inability of *V. parvula* to grow on succinate as sole carbon source and the ability of *V. parvula* to ferment lactate and cometabolize succinate can be exploited; veillonellae convert succinate exclusively to propionate during exponential and stationary growth phases (Samuelov et al., 1990). Succinate decarboxylation to propionate follows a nongrowth-linked pattern, and fermentation of

lactate to propionate follows a growth-linked pattern. Thus, a two-stage continuous culture process involving growth on lactate in the first stage enhances the production of propionate in the second stage by feeding succinate to the effluent broth from the first stage.

Veillonellae have been used to control the growth of *Salmonella typhimurium* and *Salmonella enteritidis* in vitro (Hinton and Hume, 1995). Various ratios of lactate and succinate were used in an agar overlay assay system, and the greatest inhibition was noted when the highest concentration (175 μmol) of both succinate and lactate were used. The inhibition may be due to acidic pH and volatile fatty acids produced by veillonellae. The ability of veillonellae to produce acetate and propionate end products may contribute to the effectiveness of veillonellae-containing probiotic cultures fed to chicks to reduce colonization by salmonellae. *Veillonella* strain CF3, isolated from chicken cecum, multiplied and survived in propionate concentrations of 100 mM or less, and the veillonellae increased in numbers higher than the control environment without propionate, suggesting that veillonella CF3 may possess adaptive mechanisms useful in probiotic cultures fed to chickens (Kwon et al., 1997). The production of acetate and propionate by veillonella strain AU2 from growth on tartrate inhibited the growth of *Listeria monocytogenes*, a Gram-positive food-borne pathogen (Hinton and Hume, 1997).

Reduction of methanogenesis is a way to increase animal weight because methane formed in the rumen represents a loss of energy for the animal. Efforts to improve animal performance have included the addition of fumarate to ruminant feed because many rumen bacteria including *V. parvula* oxidize H_2 by using fumarate as a final electron acceptor and forming primarily propionate as end product (Asanuma et al., 1999). Since H_2 is the main substrate for methane production, these results suggest that the fumarate-utilizing bacteria may compete with the methanogens. The fumarate-utilizing bacteria utilize malate and form similar end products as found with fumarate fermentation. Many of these bacteria including *V. parvula* utilize formate, another substrate for methanogenesis, as an electron donor for fumarate reduction (Asanuma et al., 1999).

Four species isolated from conventional rats were introduced into germ free rats to evaluate the ability of the multispecies consortium to ferment uncooked, amylomaize starch granules that contain a resistant starch fraction composed of a resistant outer shell protecting the granule against chemical or enzymatic degradation (Pacheco-Delahaye et al., 1994). The four-species consortium consisted of a *Peptostrepto-coccus* spp. (non-amylolytic, glucose fermenter), *Eubacterium* spp. (amylolytic), *Enterococcus faecalis* (non-amylolytic, able to hydrolyze maltose), and *V. ratti* (non-amylolytic, able to hydrolyze maltose and maltodextrins). In germ-free rats, only 70% of the amylomaize starch was digested; monospecies associated rats with the non-amylolytic strains *Peptostreptococcus* sp., *Enterococcus faecalis*, or *V. ratti* digested no more amylomaize starch than the germ-free rats, monospecies-associated rats with the amylolytic *Eubacterium* spp. digested up to 92%, but the four-species consortium digested 97%. In the cecum of the four-species associated rats, succinic, propionic and acetic acids increased and D-lactic acid disappeared compared to the cecum contents of germ-free or monospecies-associated rats (Pacheco-Delahaye et al., 1994). These results suggest that *V. ratti* hydrolyzed and fermented maltose and maltodextrins and utilized lactic acid end products of the other members of the consortium.

Adherence

Nichrome Steel Wire Model

The first observation that veillonellae could participate in a special adherence arrangement with other oral bacteria involved the relationship between *V. parvula* V5 (formerly *V. alcalescens*) and *Actinomyces viscosus* T6 (formerly *Odontomyces viscosus*) on nichrome steel wires that were suspended in broth cultures (Bladen et al., 1970). The veillonellae were unable to adhere to the solid supports, whereas the actinomyces growing in sucrose-containing medium formed monospecies biofilms on the wires. Transfer of the biofilm-containing wires to lactate-based medium inoculated with *V. parvula* resulted in a large increase in the amount of biofilm deposited because the veillonellae grew in the lactate medium and were apparently able to attach to the initial colonizers.

The requirement for sucrose during monospecies biofilm formation suggests that glucosyltransferase, an extracellular enzyme that catalyzes the incorporation of the glucose moiety from sucrose into a water-insoluble polysaccharide polymer (Robrish et al., 1972), may be involved in biofilm formation and that direct cell-to-cell contact may not be necessary. The enzyme is elaborated by many oral streptococci including *S. salivarius*, whose extracellular glucosyltransferase can bind directly to *V. parvula* cells (Wittenberger et al., 1977). Such enzyme-bound veillonellae can adhere in large numbers to the smooth wire surface in the presence of sucrose and in the absence of other organisms

(McCabe and Donkersloot, 1977). Thus, adherence of a cell type (veillonella) with no innate ability to adhere to a smooth surface can be effected by a secreted enzyme that is produced by a different cell type (streptococcus) and that catalyzes the formation of an insoluble, adherence-mediating polysaccharide.

Intergeneric Coaggregation

PARTNERS Direct adherence between veillonellae and oral bacteria of other genera was first demonstrated by Gibbons and Nygaard, 1970 who noted coaggregation between *V. parvula* V5 (formerly *V. alcalescens*) and *A. viscosus* T6, coccobacillus 26, and *Neisseria* 17. *V. parvula* V1 (formerly *V. alcalescens*) and *V. parvula* V4 were coaggregation partners of many of the 46 human oral *Streptococcus salivarius* isolates examined for other adherence properties (Weerkamp and McBride, 1980). Coaggregation between *V. parvula* V1 and fibrillar strains of *S. salivarius* was much stronger than with fimbriate strains of *S. salivarius* after one hour of incubation, but after 24 hours both interactions were equally strong (Handley et al., 1987). A bald mutant, *S. salivarius* HB-B, devoid of surface structures was unable to coaggregate with *V. parvula* V1 (Harty and Handley, 1988). Coculturing of *V. parvula* and *Eubacterium saburreum* resulted in cocci-filament associations that remained adherent after vigorous vortex mixing (Mashimo et al., 1981).

In a survey of nearly 200 isolates of veillonella spp., all coaggregated with *Fusobacterium nucleatum*; some coaggregated with *Actinomyces israelii*, *Actinomyces naeslundii*, *Actinomyces odontolyticus*, *Gemella morbillorum*, *Streptococcus salivarius*, *Streptococcus sanguis*, *Rothia dentocariosa*, and *Propionibacterium acnes* (Hughes et al., 1988). The patterns of coaggregation between the veillonellae and their partners delineated four coaggregation groups. None of the veillonellae coaggregated with other veillonellae, which illustrates the predilection for intergeneric coaggregation rather than intrageneric interactions.

MECHANISMS Many of the intergeneric coaggregations are inhibited by lactose (60 mM) and all of the coaggregations with streptococci and actinomyces are prevented by heat (85°C for 30 min) or protease treatment of the veillonella (Hughes et al., 1988). The same treatment of the partner has no effect on the ability of that partner to coaggregate. On the basis of the properties of numerous other coaggregations among oral bacteria (Kolenbrander, 1988a), it is likely that many coaggregations involving veillonellae are mediated by lectin-carbohydrate interactions.

For example, a lactose-sensitive lectin (protease-sensitive surface component) is expressed on the veillonella surface, and its cognate carbohydrate receptor is present on the partner surface.

V. atypica PK1910 coaggregates with oral streptococci by two mechanisms. It coaggregates with some streptococci by a lactose-inhibitable mechanism and with other streptococci by a lactose-noninhibitable mechanism. Coaggregation-defective mutants that specifically lack either or both of these mechanisms with oral streptococci have been isolated (Hughes et al., 1990). Three classes of mutants were isolated. The first class fails to participate in lactose-inhibitable coaggregations with streptococci. The second class fails to coaggregate by the lactose-noninhibitable interactions. The third class, isolated from the first class, fails to coaggregate with any oral streptococci. Two distinct surface molecules mediating either lactose-inhibitable or lactose-noninhibitable coaggregations are sufficient to account for all three types of interactions. The lactose-inhibitable interaction appears to be mediated by a 45-kDa surface protein (Hughes et al., 1992). The protein is present in surface protein preparations of the parent and class 2 mutants but is absent in class 1 and class 3 mutant surface protein preparations. It binds to agarose-lactose beads and is eluted by 100 mM lactose. The putative adhesin does not seem to be the structural subunit of veillonella fimbriae, since no differences in fimbriae were observed by electron microscopy of the parent and all three classes of mutants (Hughes et al., 1992). It is likely that the 45-kDa surface protein is an adhesin that binds at or near the tip of the fimbrial structure as has been observed for a lactose-sensitive adhesin in another human oral Gram-negative bacterium, *Prevotella loescheii* (formerly *Bacteroides loescheii*; London and Allen, 1990; Weiss et al., 1988).

RELATIONSHIP TO COLONIZATION SITE Fifty eight of 59 veillonella isolates obtained from the human tongue dorsum coaggregated with *S. salivarius*, a predominant inhabitant of the tongue (Hughes et al., 1988). However, none of the 58 tongue isolates, which were all *V. atypica* or *V. dispar*, coaggregated with *Actinomyces naeslundii*, *Actinomyces israelii*, and *Streptococcus sanguis* isolated from subgingival plaque. In contrast, 24 subgingival veillonellae, of which 20 were *V. parvula*, coaggregated with the actinomyces, streptococci and other normal inhabitants of subgingival plaque, but they exhibited no coaggregation with strains of *S. salivarius*. Five other subgingival veillonellae failed to coaggregate with subgingival bacteria but did coaggregate with *S. salivarius*. Greater than 80% (87 of 105 strains) of the veillonellae isolated from

buccal mucosa and saliva coaggregated with *S. salivarius*, the predominant streptococcus found in such samples. These results indicate the potential for highly specific, spatiotemporally distinct interbacterial adherence. On the basis of the timing of appearance of various bacteria after tooth eruption and increased age of the host, spatiotemporal adherence is likely to mediate bacterial colonization of different habitats within the oral cavity.

An early observation of interbacterial interactions was the presence of "corn cob" and "test tube brush" morphologies of bacteria in dental plaque (Jones, 1972; Listgarten, 1976). A central rod or filamentous cell was surrounded by clusters of spherical cells in the former and by shorter rods in the latter. These cellular arrangements were commonly observed at the edges of developing plaque rather than in the center or near the tooth surface. Veillonellae easily form corncob structures with fusobacteria when the numbers of veillonellae are in large excess (Fig. 2A). When the numbers of the two cell types are equal, large coaggregates are formed not only with fusobacteria (Fig. 2B), but also with streptococci (Fig. 2C), and actinomyces (Fig. 2D). In each pairing the large coaggregates are composed of an interacting network of both cell types. Smaller coaggregates are visible (large arrows, Fig. 2C and 2D) and even when the partners have the same shape (Fig. 2C), the interaction between the two cell types is clearly seen.
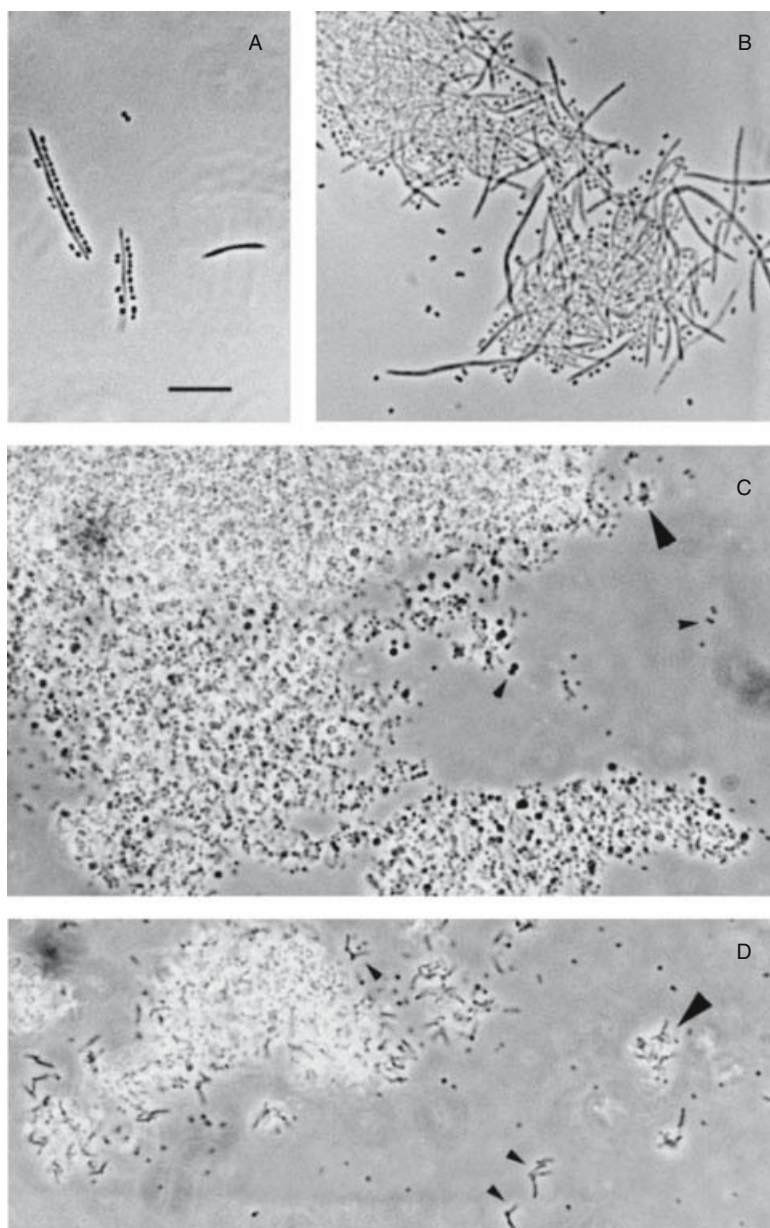
Fig. 2. Intergeneric coaggregation between *Veillonella atypica* PK1910 and other oral bacteria. When the ratio of *Veillonella atypica* PK1910 to *Fusobacterium nucleatum* PK1594 is 10 to 1, (A) coaggregates appear as "corn cobs" where the coccal-shaped veillonellae line up along the length of the slender rod-shaped fusobacteria. Coaggregates appear as clumps of mixed cell-types when equal numbers of: (B) fusobacteria, (C) *Streptococcus oralis* (formerly *S. sanguis*) 34, or (D) *Actinomyces naeslundii* (formerly *A. viscosus*) T14V are combined with the veillonellae. The large arrows in Figs. 2C and 2D indicate small coaggregates composed of a few cells of each cell type; the small arrows in Fig. 2C indicate the large phase-dark streptococcal spheres and the smaller and lighter gray veillonellae; and the small arrows in Fig. 2D indicate the characteristic V-shaped or irregularly shaped actinomyces. Bar = 10 μm. All figures are at the same magnification.

The oral cavity is a lotic environment, which selects for adherent inhabitants. Saliva lubricates and cleanses the mouth and it contains lysozyme and bicarbonate ions that can lyse veillonellae (Tortosa et al., 1981). Adherence to other already attached bacteria on the tooth or mucosal surfaces may protect veillonellae from these lytic activities as well as be their primary means of accretion. For example, a cell surrounded by coaggregation partner cells of a different genus is sequestered and is unable to coaggregate with other bacteria (Kolenbrander and Andersen, 1988b). Likewise, the central cell of a rosette may be protected from harmful molecules by having the outer cells of the rosette inactivate them.

Gnotobiotic Animal Model

Coaggregation in vivo between *V. parvula* V-1 (formerly *V. alcalescens*) and *S. mutans* was demonstrated with a gnotobiotic rat model system (McBride and van der Hoeven, 1981). The *Veillonella* strain could not colonize the teeth but the two strains of *S. mutans* attached and colonized the smooth tooth surface. The veillonellae coaggregated with only one of the strains of *S. mutans*. If the coaggregation-positive strain of *S. mutans* was allowed to colonize the teeth before infection with the veillonellae, then the veillonellae attached and colonized the tooth surface. In contrast, no colonization of veillonellae above control values was seen when the coaggregation-negative *S. mutans* was used. The validity of the animal model was supported by the results of administering another coaggregating pair, whose adherence could be inhibited by lactose (van der Hoeven et al., 1985). One member of the pair was allowed to colonize. Its partner was then added to the lactose-containing drinking water. This reduced the initial adherence of the coaggregating partner to control levels found when the partner was given alone to the animal. These results indicate that intergeneric coaggregation among oral bacteria is sensitive in vivo to similar perturbations (e.g., lactose inhibition) in the gnotobiotic animal as is found in vitro by mixing suspensions of the two partner strains (Kolenbrander, 1988a; Kolenbrander and Andersen, 1986).

Earlier studies had demonstrated that human strains of *V. parvula* (formerly *V. alcalescens*) could not establish in mono-infected germ-free mice, whereas *Streptococcus mitis* could colonize by itself (Gibbons et al., 1964). However, when inoculated with several other human strains including *S. mitis*, *V. parvula* readily became part of the oral flora, suggesting that the veillonellae adhere to primary colonizers but not directly to the host tissues. It is also possible that lactic acid

is in limiting supply in the gnotobiotic animal and that veillonellae colonize poorly in the absence of an adequate nutrient supply. In either situation veillonellae require other bacteria to establish themselves in the oral cavity.

Human Oral Cavity

Veillonellae are considered to be among the early colonizers of freshly cleaned enamel although they adhere poorly to a cleaned, human tooth surface (Liljemark and Gibbons, 1971) and to spheroidal hydroxyapatite (McBride and van der Hoeven, 1981), an extensively used model surface for adherence of oral bacteria to in vivo enamel surfaces (Clark et al., 1978). Their numbers increase in parallel with the numbers of actinomyces and after the streptococci are established (Ritz, 1967). Their inability to adhere well to smooth surfaces is in sharp contrast to their ability to recognize the surface of initial adherent cells such as *S. sanguis*, *A. naeslundii*, and *A. israelii*, which are known to bind to spheroidal hydroxyapatite (Clark et al., 1978; Clark et al., 1981; Kolenbrander and Celesk, 1983).

Veillonellae are found in proportionally very high numbers on the tongue dorsum as compared to their numbers in buccal mucosa samples or dental plaque (Liljemark and Gibbons, 1971). In this study streptomycin-resistant strains of *Veillonella* were introduced into the mouths of human volunteers for 5 minutes. Samples were taken from the tongue dorsum and tooth surface after 45 minutes and plated on streptomycin-containing agar (Rogosa et al., 1958), and the resulting colonies (number of veillonellae) were counted. The distribution of veillonellae on the two surfaces was according to their proportions found naturally.

The ecological relationship of coaggregation to colonization site was examined by testing veillonellae from subgingival plaque or the dorsum of the tongue for their ability to coaggregate with other bacteria isolated from the tongue or from subgingival plaque (Hughes et al., 1988). Results from that study indicate that bacteria occupy the same site as their coaggregation partners and suggest a direct link between coaggregation and colonization in the oral cavity.

Bacteriophage

Both temperate (Shimizu, 1968) and virulent (Totsuka, 1976) phages for human oral veillonellae have been isolated. The phage receptor for one of the veillonellophages is lipopolysaccharide (Totsuka, 1988; Totsuka and Ono, 1989).

PLASMIDS Plasmids are found in about 50% of the human oral *Veillonella* strains and most have

several plasmids whose molecular sizes range from 1.1 to 28 MDa (Arai et al., 1984). It has been suggested that fructose fermentation by some strains of *V. criceti* may be associated with plasmid-encoded genes (Mays et al., 1982).

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CHAPTER 1.3.7

Syntrophomonadaceae

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Introduction

The family Syntrophomonadaceae includes a group of anaerobes that commonly grow in syntrophic associations with molecular hydrogen (H_2)- and formate-using methanogens. The energy source for these organisms is the oxidation of carboxylic acids of four carbons or more. This oxidation is normally coupled to the production of H_2 or formate, which is consumed by methanogens or other organisms. The Syntrophomonadaceae are phylogenetically a part of the phylum of Gram-positive bacteria with low DNA G+C content. Although many of the members of this family have cell walls typical of Gram-positive bacteria, several genera *(Syntrophomonas, Thermosyntropha, Pelospora* and *Syntrophothermus*) have cell walls typical of Gram-negative bacteria, with the outer membrane of *Syntrophomonas* confirmed by electron microscopy. The division between Gram-negative and Gram-positive microbes has historically been considered an indicator of the deepest taxonomic separations (Buchanan and Gibbons, 1974; Gibbons and Murray, 1978), so it is unusual to find members of the same family containing both of these cell wall structures. Another example of this can be found in the family Acidaminococcaceae, also a family of low-G+C Gram-positive bacteria, that contains some genera which have ultrastructures typical of Gram-negative bacteria.

In general, the members of this family are unified by their common environmental association with methanogens, and this association makes their metabolic capabilities possible. These bacteria are found predominantly in anaerobic environments where organic matter is completely degraded to methane and carbon dioxide. In such environments, oxygen, sulfate, nitrate, nitrite, and ferric ions are not readily available as electron acceptors for the degradation of organic matter; thus methanogenesis is the dominant process. Within these ecosystems, members of the family are found in syntrophic associations with methanogens.

Whereas most of the members of Syntrophomonadaceae grow by syntrophically oxidizing monocarboxylic acids, *Pelospora* differ. They do not require syntrophy (they ferment succinate or glutarate) and they are found in nonmethanogenic environments. The other members of this family obtain their energy for growth from the degradation of a variety of fatty acids ranging from four to eighteen carbons in length. The oxidation of these compounds is thermodynamically unfavorable unless the products (H_2 and/or formate) are maintained at low concentrations by the action of the syntrophic partner, such as a methanogen (Table 1). When the methanogenic partner is absent, the concentrations of H_2 and formate rapidly increase to values that thermodynamically inhibit fatty-acid oxidation. Therefore, syntrophic bacteria in their natural environment are obligately dependent on the activity of the methanogens. There are no alternate fermentative pathways for energy production from compounds such as butyrate. When these organisms first were isolated, no mechanism other than syntrophy was known that would allow their growth, and they were called "obligate proton-reducing acetigens" (McInerney et al., 1979). Since that time, butyrate-degrading syntrophs were found capable of growth on crotonate (Beaty and McInerney, 1987). This eliminates the need for a H_2/formate-using bacterium, allowing their growth as pure cultures.

Phylogeny and Taxonomy

Sequence analysis of the 16S rDNA genes (Fig. 1) indicates that the family Syntrophomonadaceae is monophyletic, and separate from the genera *Moorella* and *Thermoanaerobacter*. The family contains five genera, most of which are also monophyletic. However, the genus *Syntrophomonas* appears to be paraphyletic. In 84 of 100 bootstrap trees, *Syntrophomonas wolfei*, the type species of *Syntrophomonas*, is more closely related to *Syntrophospora bryantii* and *Pelospora glutarica* than it is to *Syntrophomonas sapovorans*. The phylogenetic distance between *Syntrophomonas sapovorans* and *Syntrophomonas wolfei* is sufficient (sequence similarity

Table 1. The major reactions involved in the anaerobic degradation of mono- and dicarboxylic acids by members of the family Syntrophomonadaceae.

Reactions	Free energy yield $\Delta G^{o\prime}$ (kJ/mol)
Some reactions of H$_2$/formate-using bacteria	
Methanogens	
$4H_2 + HCO_3^- + H^+ \leftrightarrow CH_4 + 3H_2O$	−135.6
$4HCO_2^- + H_2O + H^+ \leftrightarrow CH_4 + 3HCO_3^-$	−130.4
Sulfate-reducing bacteria	
$4H_2 + SO_4^{2-} + H^+ \leftrightarrow HS^- + 4H_2O$	−151.0
Some reactions of members in the family Syntrophomonadaceae:	
Without H$_2$/formate-using bacterium	
$CH_3CH_2CH_2COO^- + 2H_2O \leftrightarrow 2CH_3COO^- + H^+ + 2H_2$	+48.1
$CH_3CH_2CH_2CH_2COO^- + 2H_2O \leftrightarrow CH_3COO^- + CH_3CH_2COO^- + H^+ + 2H_2$	+48.1
$2CH_3HC=CH—COO^- + 2H_2O \leftrightarrow 2CH_3COO^- + CH_3CH_2CH_2COO^- + H^+$	−101.9[a]
$^-OOCCH_2CH_2COO^- + H_2O \leftrightarrow CH_3CH_2COO^- + HCO_3^-$	−20.5[b]
With H$_2$/formate-using bacterium	
$2CH_3CH_2CH_2COO^- + HCO_3^- + H_2O \leftrightarrow 4CH_3COO^- + CH_4 + H^+$	−39.4
$2CH_3CH_2CH_2CH_2COO^- + HCO_3^- + H_2O \leftrightarrow 2CH_3COO^- + 2CH_3CH_2COO^- + CH_4 + H^+$	−39.4
$2CH_3CH_2CH_2COO^- + SO_4^{2-} \leftrightarrow 4CH_3COO^- + HS^- + H^+$	−54.8
$2CH_3CH_2CH_2CH_2COO^- + SO_4^{2-} \leftrightarrow 2CH_3COO^- + 2CH_3CH_2COO^- + HS^- + H^+$	−54.8

[a]Most members of the family Syntrophomonadaceae grow on crotonate in pure culture.
[b]*Pelospora glutarica* is the only member of the Syntrophomonadaceae family that is capable in growing on succinate.
Data compiled from Schink (1997) and Thauer et al. (1977), or were calculated from data therein.
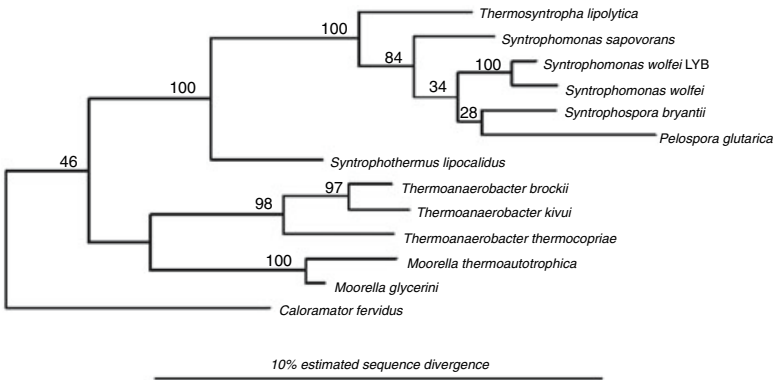
Fig. 1. Phylogenetic tree for members of the Syntrophomonadaceae family and related organisms based upon 16S rDNA sequences. The GenBank accession numbers for the 16S rRNA sequences reported in this paper are as follows: *Thermosyntropha lipolytica* DSM 11003[T] {X99980}, *Pelospora glutarica* WoGI3[T] {AJ251214}, *Syntrophospora bryantii* DSM 3943[T] {M26491}, *Syntrophomonas sapovorans* DSM 3441[T] {AF022249}, *Syntrophomonas wolfei* subsp. *wolfei* DSM 2245[T] {M26492}, *Syntrophomonas wolfei* LYB {AF022248}, *Syntrophothermus lipocalidus* TGB-C1[T] {AB021305}, *Moorella thermoautotrophica* DSM 1974[T] {X77849}, *Moorella glycerini* DSM 11254[T] {482327}, *Caloramator fervidus* ATCC 43204[T] {L09187} *Thermoanaerobacter thermocopriae* IAM 13577[T] {L09167}, *Thermoanaerobacter brockii* HTD4[T] {L09165}, and *Thermoanaerobacter kivui* DSM 2030[T] {L09160}. Sequences were manually aligned and checked for secondary structure to ensure only homologous regions were compared. The aligned sequences included 1336 bases, but a mask (Page and Holmes, 1998) was employed that reduced the number of nucleotides that were compared to 1197. Evolutionary distances were computed from pairwise similarities by employing the correction of Jukes and Cantor (1969). The program ARB was used for analyzing and assembling sequence data. The phylogenetic trees were constructed by using the maximum-likelihood analysis with fastDNAml (Olsen et al., 1994). Bootstrap values were determined for the maximum-likelihood trees with 100 replicates.

determined from a binary comparison of 16S rDNA genes of these two species is 92%, Matthies et al., 2000, or 95% in our masked alignment) to support the proposal of a separate genus within the family for *Syntrophomonas sapovorans*.

Habitat

Members of the family Syntrophomonadaceae are predominantly found in methanogenic environments in syntrophic associations with methanogens. In such environments, various electron acceptors are not readily available and methanogenesis is the dominant metabolism (McInerney, 1986). In such environments, primary fermentative bacteria degrade organic matter to extracellular products including H_2, formate and acetate. Each of these is consumed directly by methanogens in the production of methane and carbon dioxide. Other products of the primary fermentative bacteria, such as monocarboxylic acids, cannot be used directly by methanogens. Rather, these compounds are degraded by syntrophic interactions between methanogens and syntrophs such as Syntrophomonadaceae. The important syntrophs that degrade fatty acids between 4 and 18 carbons are in this family, whereas those that degrade propionate (3 carbons) belong in the genera *Syntrophobacter* (Boone and Bryant, 1980) or *Smithella* (Liu et al., 1999).

Thus, syntrophic bacteria represent an essential trophic level that convert the fatty acids produced by fermentative bacteria from complex organic matter to methanogenic substrates such as acetate, H_2 and formate. Examples of such environments include sewage digestors, waterlogged soils, aquifers and sediments. Syntrophic bacteria are generally found where organic matter is degraded and inorganic electron acceptors are absent. In shallow marine sediments, sulfate reduction is the dominant metabolism, and many fatty acids may be degraded directly by sulfate-reducing bacteria. However, syntrophy does still occur in such environments (Stieb and Schink, 1985; Tschech and Schink, 1985a; 1985b).

Syntrophomonadaceae can also grow in nonmethanogenic anoxic environments: *Pelospora glutarica* is able to grow on compounds such as succinate in the absence of a syntrophic partner (Matthies et al., 2000).

Isolation

The species of Syntrophomonadaceae are extremely sensitive to oxygen. Stringent anaerobic techniques as described by Hungate (1969) or modifications of those techniques (Sowers and Noll, 1995) are used to prepare anaerobic media and solutions for cultivation. These methods usually involve the replacement of air with oxygen-free gases and the addition of strong chemical reducing agents such as cysteine, mercaptoethanesulfonate, and sodium sulfide (alone or in combinations) to maintain low oxidation-reduction potentials.

Selective Enrichment

Syntrophomonadaceae often grow in the presence of large numbers of heterotrophic bacteria. Therefore, it is difficult to isolate them directly without a preliminary enrichment step. Fatty acid-degrading syntrophs can be selectively enriched in a reduced medium that contains a fatty acid as electron donor and carbon dioxide as electron acceptor. Oxygen, ferric iron, sulfate, sulfur, nitrate, and nitrite should be avoided because such ions would support the growth of nonsyntrophic bacteria. The culture must contain a suitable H_2-utilizing methanogen to maintain low concentrations of hydrogen and make the degradation of fatty acids thermodynamically favorable. Such methanogens may be present in starting sample material or a suitable methanogen may be added.

Enrichment media, like the media for cultivation of pure cultures of syntrophs, are usually buffered by carbon dioxide and bicarbonate to a pH similar to that of the environment, and contain inorganic ions. A small amount of organic compounds that may include possible growth factors is often added. Rumen fluid (2–20% of volume) or yeast extract and peptones (0.05–0.5 g) are sometimes added for this purpose. However, additions of organic matter other than the syntrophic substrate may support the growth of nonsyntrophic bacteria, so these should be minimized. Additionally, the use of organic reducing agents should be avoided. The use of cysteine or mercaptoethanesulfonate may enrich for bacteria that degrade these compounds. The catabolic substrate is added at a concentration of about 1–2 g per liter. Certain species of the Syntrophomonadaceae family can be selected for by addition of a specific substrate. For instance, *Syntrophothermus lipocalidus* uses isobutyrate, which no other species in the family can metabolize.

To obtain numerically important syntrophs from an environment, dilutions of the original sample are inoculated into enrichment media and incubated. After a suitable time of incubation, the highest dilution (smallest inoculum) that produced a successful enrichment culture should contain the syntrophic organism that was most numerous in the original sample. This enrichment culture may be used as inoculum to isolate syntrophic cocultures.

Growth Media and Solutions

A large number of anoxic media have been formulated for the growth of fatty-acid-degrading syntrophic bacteria. Many are found in the original species description papers cited throughout this chapter. We describe only a generalized medium here. Optimal growth of these microorganisms may require modification of the concentrations of some of the components or special additions. The following basal medium can be used to cultivate the various species of the family Syntrophomonadaceae in cocultures by changing the catabolic substrate added to the medium (McInerney et al., 1979):

Basal Medium

Mineral solution (see below)	5%
Trace metal solution (see below)	0.1%
Vitamin solution (see below)	0.5%
Rumen fluid	2%
Sulfide-reducing solution (see below)	2%
Coenzyme M	0.05%
Resazurin	0.0001%
Substrate	0.2%

The bicarbonate medium is prepared by dissolving NaOH (3.5 g per l liter) in oxygen-free distilled water and equilibrating the preparation with 100% carbon dioxide. All the constituents above (except the sulfide-reducing solution) are added at the indicated final concentrations in percent (v/v), and the pH of the medium is adjusted to 7.2–7.4 under a stream of oxygen-free N_2 and CO_2 (4 : 1) gas mixture. The medium is then dispensed (under continuous stream of the above anoxic gas mixture) into 27-ml serum tubes fitted with butyl rubber stoppers, sealed, and autoclaved (121°C, 20 min). The medium is cooled, and before use, the sulfide-reducing solution is added individually to each tube. For solid media, purified agar (18 g per l liter) is added to the medium and is maintained in suspension by utilizing a magnetic stirrer as it is dispensed into serum tubes.

Mineral Solution

KH_2PO_4	10.0 g
$MgCl_2 \cdot 6H_2O$	6.6 g
NaCl	8.0 g
NH_4Cl	8.0 g
$CaCl_2 \cdot 2H_2O$	1.0 g
Distilled water	1 liter

Trace Metal Solution

Nitrilotriacetic acid	2.0 g
$MnSO_4 \cdot H_2O$	1.0 g
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.8 g
$CoCl_2 \cdot 6H_2O$	0.2 g
$ZnSO_4 \cdot 7H_2O$	0.2 g
$CuCl_2 \cdot 2H_2O$	0.02 g
$NiCl_2 \cdot 6H_2O$	0.02 g
$Na_2MoO_4 \cdot 2H_2O$	0.02 g
Na_2SeO_4	0.02 g
Na_2WO_4	0.02 g
Distilled water	1 liter

To prepare the trace metal solution, dissolve the nitrilotriacetic acid in 800 ml of distilled water and adjust the pH to 6.0 with KOH. Then dissolve the minerals and bring the volume to 1 liter.

Vitamin Solution

Nicotinic acid	20 mg
Cyanocobalamin	20 mg
Thiamin · HCl	10 mg
p-Aminobenzoic acid	10 mg
Pyridoxine · HCl	50 mg
Calcium pantothenate	5 mg
Distilled water	1 liter

The substrate is added at a concentration, which will not inhibit growth. For short-chain fatty acids, a concentration of 20 mM is used, while lower concentrations (>5 mM) are used for long-chain fatty acids. Higher concentrations of long-chain fatty acids require the supplementation of equimolar calcium chloride into the medium.

For growth of lipolytic, alkalitolerant, fatty acid degrading bacteria, the medium is prepared under a nitrogen atmosphere (pH 8.2) and may be supplemented with olive oil (20 ml per liter) and yeast extract (5 g per liter) rather than rumen fluid (Svetlitshnyi et al., 1996).

Culturing H_2-Utilizing Microorganisms

Several mesophilic anaerobes that syntrophically oxidize fatty acids have been isolated in cocultures with H_2-utilizing strains such as *Desulfovibrio* and *Methanospirillum hungateii*. *Syntrophomonas wolfei* subsp. *wolfei* was the first documented mesophilic strain isolated in coculture with a methanogen or a sulfate-reducer. In addition, thermophilic fatty acid-oxidizing bacteria such as *Thermosyntropha lipolytica* and *Syntrophothermus lipocalidus* have been obtained in cocultures with strains of *Methanobacterium thermautotrophicum* or related strains. Thus, choosing the appropriate H_2-utilizing microbe is essential to obtain cocultures of syntrophic bacteria. Fatty acid β-oxidation tends to be thermodynamically more favorable when it is coupled to H_2 utilization by a sulfate-reducer rather than by a methanogen because the reduction of sulfate to sulfide by H_2 is thermodynamically more favorable than the reduction of CO_2 to CH_4 by H_2. Thus, sulfate-reducing, syntrophic cocultures grow faster and to higher yields than the methanogenic cocultures, but it may be easier later to obtain axenic cultures when methanogens are used as partners.

The basal medium above without the addition of a substrate may be sufficient for the growth of the H_2-utilizing microorganisms. A gas mixture of H_2 and CO_2 (4 : 1) is added after dispensing and autoclaving the medium. The culture tubes are pressurized to 100 kPa above atmospheric pressure. For the growth of H_2-utilizing sulfate-

reducing bacteria, the basal medium is supplemented with 3 g of Na_2SO_4.

During growth on H_2 and CO_2, methanogens quickly consume gas to produce CH_4 (5 mol of gas: 1 mol of CH_4); therefore a negative pressure develops in the culture tubes. Additionally, as the partial pressure of CO_2 decreases, the medium becomes alkaline, which may inhibit growth and cause cell lysis. To minimize these problems, the volume of the headspace should be pressurized periodically throughout the growth period with N_2 and CO_2 (4 : 1 vol/vol).

Isolation of Cocultures and Axenic Cultures

Serial ten-fold dilutions of the enrichment culture are prepared while avoiding exposure of the cultures to O_2, and a culture of the H_2-using partner organism is added to each tube. These tubes are mixed and inoculated into molten anaerobic roll-tube media (45°C) with and without the catabolic substrate. The roll tubes are rolled to coat the agar on the inside of the tube and incubated at an appropriate temperature. Colonies may take several months to develop, and result in syntrophic cocultures. Colonies that appear within the first few weeks of incubation are probably growing too fast to be syntrophic cocultures, and these may be marked so that late-forming colonies can be easily distinguished. Syntrophic colonies contain a mixture of the syntroph and its partner, so when a methanogen is used as the syntrophic partner, the syntrophic colonies may be distinguished by the presence of the methanogens' epifluorescence. Colonies are selected, picked, and inoculated into enrichment medium. These cultures are immediately diluted and re-inoculated into roll-tube media together with the syntrophic partner (as described above). This process is repeated until a single colony type remains. At this point, the culture contains a single strain of syntrophic bacteria plus the syntrophic partner that was selected.

To obtain axenic cultures from these cocultures, it is necessary to find a suitable substrate that supports the growth of the syntroph without its partner. Crotonate has been successfully used for butyrate-degrading bacteria. The coculture is grown in medium with crotonate as the sole catabolic substrate; this enriches for the butyrate-degrading syntroph and limits the growth of the methanogen. A pure culture of the syntroph may be obtained by serial dilution of the culture (with higher dilutions having the syntroph present but no methanogens) or by preparing roll tube media with crotonate to obtain pure colonies of the syntroph.

Maintenance Procedures

Pure cultures or cocultures may be stored for several weeks as liquid suspensions, or suspensions can be frozen in the presence of cryoprotectant (5% glycerol) by cooling at 1°C/min and stored at liquid nitrogen temperatures (Boone, 1995), or cultures can be maintained by regular subculturing (Hippe, 1984).

Identification

Syntrophomonadaceae includes organisms that oxidize monocarboxylic acids with 4–18 carbons syntrophically, and are unable to use alternate electron acceptors such as sulfate. The descriptions below include some of the most important and distinctive characteristics of the genera, and more detailed descriptions may be found in the original citations, which are included in the footnotes to Table 2. Whenever possible, the descriptions of the genera summarize the properties of all the described species as well as the type species. The description of many of the species of this family is based on a single strain. Additionally, caution must be exercised in the evaluation of results from different laboratories because many of the growth descriptions depend greatly upon experimental conditions. In particular, variation in growth optima may occur depending on whether growth is measured by turbidity, cell counts, methane formation, growth rate, or growth yield.

Pelospora

Only one species, the type species *Pelospora glutarica*, has been described. The ability to grow on dicarboxylic acids such as glutarate, methylsuccinate, and succinate, without syntrophic interactions is the most distinctive and perhaps the most important phenotypic feature of *Pelospora*. No other genus in the family metabolizes such substrates either alone or in syntrophic associations. The rod-shaped cells of *Pelospora* stain Gram negative, are motile by one subpolar flagellum, and form terminal endospores. This mesophilic organism grows most rapidly at pH values of 7.1–8.2 and salt concentrations lower than 100 mM.

Syntrophomonas

Members of the genus *Syntrophomonas* contain Gram-negative, nonsporeforming, weakly motile, rod-shaped cells. These cells possess 2–8 flagella that are laterally inserted in a linear fashion on the concave side of the cell about 130 nm or more apart. The species and subspecies of *Syntrophomonas* are differentiated from each

Table 2. Phenotypic characteristics of members in the family Syntrophomonadaceae.

Characteristic	Pelospora	Syntrophomonas	Syntrophospora	Syntrophothemus	Thermosyntropha
Morphology					
Cell shape	Rod	Rod	Rod	Rod	Rod
Cell size (μm)	0.8×4.5–6.5	0.4–0.7×2–3.7	0.4×3–6	0.4–0.5×2.0–4.0	0.3–0.4×2.0–3.5
Gram stain	−	−	+	−	−
Flagella	+	+	+	+	−
Spore formation	+	−	+	−	−
Substrates utilized					
In pure culture					
Pyruvate	−	−	−	−	+[a]
Yeast extract	−	−	−	−	+
Tryptone	−	−	−	−	+
Casamino acids	−	−	−	−	+
Crotonate	−	+/−[b]	+	+	+
Succinate	+	−	−	−	−
Glutarate	+	−	−	−	−
In coculture w/H$_2$-using bacterium:					
Triacylglycerides	−	−	−	−	+
Propionate	−	−	−	−	−
Methylbutyrate	ND	ND	+	ND	ND
Isobutyrate	−	−	−	+	−
C$_4$–C$_{10}$	−	+	+	+	+
C$_{11}$–C$_{18}$	−	+/−[c]	+	−	+
Elaidate	−	+	ND	ND	ND
Isovalerate	−	−	−	−	−
Isoheptanoate	−	+	ND	−	ND
Oleate	−	+/−[d]	ND	−	+
Linoleate	−	+/−[e]	−	−	+
Olive oil	−	ND	ND	ND	+
Growth requirements					
Organic growth factors	Rumen fluid	PABA + B-vitamins[f]	None	None	Yeast extract
Conditions supporting most rapid growth:					
Temperature (°C)	37	37–40	28–34	55	60–66
pH	7.1–8.2	5.0–7.0	6.5–7.5	6.5–7.0	8.1–8.9

Abbreviations: +, present in all species; −, absent in all species; ND, not determined; and PABA, *p*-aminobenzoic acid.
[a]This genus weakly degrades pyruvate in pure culture.
[b]Most species within this genus can grow in pure culture on crotonate, however, *Syntrophomonas sapovorans* cannot.
[c]Some species in this genus can only degrade fatty acids up to C$_{12}$.
[d]Some species in this genus cannot degrade oleate in syntrophic co-cultures.
[e]Some species in this genus cannot degrade linoleate in syntrophic co-cultures.
[f]The B-vitamins include biotin, thiamine, cyanocobalamin, and lipoic acid.
Data compiled from: Beaty and McInerney (1990); Lorowitz et al. (1989); Matthies et al. (2000); McInerney et al. (McInerney et al., 1979, McInerney et al., 1981); Roy et al. (1986); Sekiguchi et al. (2000); Stieb and Schink (1985); Svetlitshnyi et al. (1996), and Zhao et al. (Zhao et al., 1990, Zhao et al., 1993).

other on the basis of their substrate utilization patterns (Table 2). *Syntrophomonas wolfei* contains two subspecies, one of which (*S. wolfei* subsp. *wolfei*) syntrophically degrades normal saturated fatty acids with 4–8 carbons and isoheptanoate; whereas *S. wolfei* subsp. *saponavida* syntrophically degrades normal saturated fatty acids with 4–18 carbons and the *iso*-fatty acids isoheptanoate and longer. All species and subspecies of *Syntrophomonas* grow in pure culture on crotonate and are stimulated by the addition of B vitamins, amino acids, or rumen fluid. Most

rapid growth is observed at 35–37°C and at near neutral pH.

Syntrophospora

The rod-shaped cells of *Syntrophospora* have ultrastructure similar to that of Gram-positive bacteria and are nonmotile. The most distinctive feature of this genus is that the cells form oval, terminal endospores during growth on fatty acids. Saturated fatty acids (butyrate and longer) are β-oxidized to acetate and H$_2$ (or also propi-

onate in the case of odd-numbered acids) in syntrophic cooperation with H_2-utilizing microorganisms. *Syntrophospora* grows in pure culture on crotonate. Most rapid growth occurs at near neutral pH and at 34°C. The type strain of this genus is *Syntrophospora bryantii*.

Syntrophothermus

The rod-shaped cells of *Syntrophothermus* stain Gram-negative, are weakly motile, and non-sporeforming. This organism can grow in pure culture only on crotonate. In syntrophic cooperation with H_2-utilizing microorganisms, cells of *Syntrophothermus* can metabolize saturated fatty acids with 4–10 carbon atoms by β-oxidation. The ability to degrade isobutyrate by isomerization to butyrate in syntrophic cooperation with an H_2-utilizing organism is the most distinguishing phenotypic feature of *Syntrophothermus*. Most rapid growth is observed at 55°C and at near neutral pH. *Syntrophothermus lipocalidus* is the type species of the genus.

Thermosyntropha

Thermosyntropha lipolytica is the type, and so far, the only species in this genus. The ability to hydrolyze triglycerides and utilize the liberated short- and long-chain fatty acids in syntrophic cooperation with an H_2-utilizing methanogen is the most distinctive and perhaps the most important phenotypic feature of *Thermosyntropha*. This organism can be cultured on a number of substrates (refer to Table 2), whereas most species in the family can only grow on crotonate in pure culture. Oleate, linoleate, and saturated fatty acids (butyrate up to stearate) are metabolized in cocultures with H_2-utilizing microorganisms. The rod-shaped cells of *Thermosyntropha* stain Gram negative, are nonmotile, and non-sporeforming. This organism grows mostly rapidly at pH 8.1–8.9 and at temperatures between 60 and 66°C.

Biochemical and Physiological Properties

Biochemistry of Syntrophic Butyrate Metabolism

Fermenation of butyrate to acetate and H_2 is an endergonic reaction (see Table 1) under standard conditions. Degradation of butyrate is feasible only at a low H_2 partial pressure (below 10^{-4} to 10^{-5} atm; McInerney et al., 1981; Schink, 1997), which can be maintained by methanogenic bacteria. The pathway of butyrate oxidation in syntrophic butyrate-oxidizing bacteria has been

tentatively elucidated with *Syntrophomonas wolfei*. The pathway proceeds through classical fatty acid β-oxidation which involves seven steps (Wofford et al., 1986). Step one involves the activation of butyrate via acetyl-CoA by CoA-transferase (Fig. 2, step 1). This is followed by the oxidation of butyryl-CoA to crotonyl-CoA via the enzyme acyl-CoA dehydrogenase (Fig. 2, step 2). The electron transfer is mediated by the flavin-adenine dinucleotide (FAD) prosthetic group of the enzyme. The formation of L(+)-3-hydroxybutyryl-CoA is catalyzed by the enoyl-CoA hydratase, which incorporates a water molecule into crotonyl-CoA (Fig. 2, step 3). Then L(+)-3-hydroxybutyryl-CoA undergoes dehydrogenation to acetoacetyl-CoA via the L(+)-3-hydroxybutyryl-CoA dehydrogenase enzyme (Fig. 2, step 4). This electron transfer is mediated by the coenzyme NAD^+. The fifth step of β-oxidation is the thiolase reaction (Fig. 2, step 5), which yields two acetyl-CoA molecules. This reaction proceeds through a Claisen ester cleavage, with the first two carbons released as acetyl-
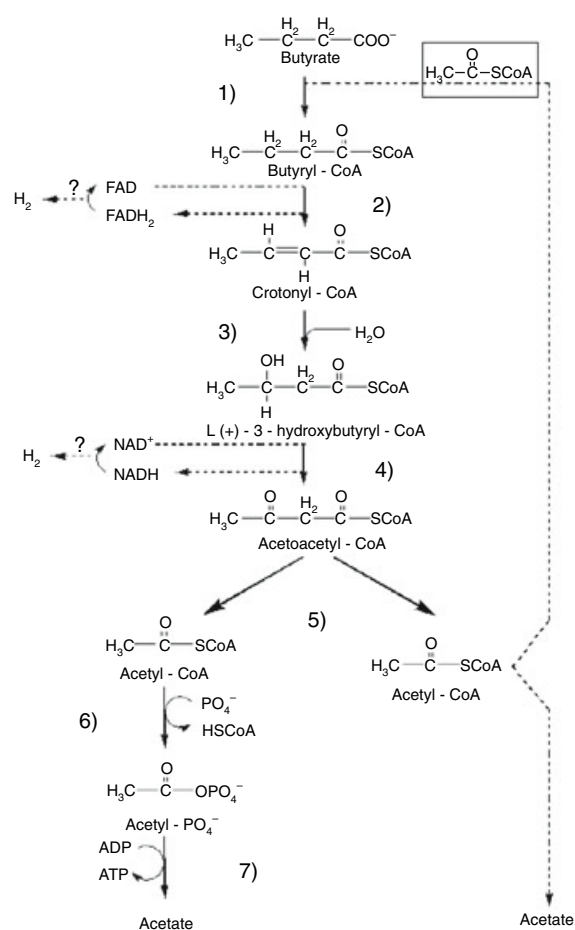
Fig. 2. Pathway of butyrate degradation by *Syntrophomonas wolfei*.

CoA and the second two carbons forming an enzyme-thioester intermediate. This acetyl group is cleaved with CoA by a displacement reaction, generating the second acetyl-CoA molecule. In step 6 (Fig. 2, step 6), acetyl-CoA is converted to acetyl-PO_4^{2-} by the enzyme phosphotransacetylase, via a displacement reaction involving inorganic phosphate. The terminal reaction, catalyzed by acetate kinase, yields an acetate molecule and one ATP by means of substrate-level phosphorylation (Fig. 2, step 7).

The proposed butyrate degradation pathway suggests that for every mole of butyrate oxidized to acetate one mole of ATP is synthesized. Thauer and Morris (1984) proposed that some of the ATP produced from substrate-level phosphorylation is used to drive the reverse electron flow, via proton motive force (Wallrabenstein and Schink, 1994), associated with H_2 production from the reaction mentioned above (Fig. 2, step 2). This reverse electron transport would be necessary for the production of H_2 from FAD. Such reversed electron transport processes have been observed with cell suspensions of *Methanosarcina barkeri* (Bott and Thauer, 1989) and *Desulfovibrio vulgaris* (Pankhania et al., 1988). This is consistent with the amount of ATP anticipated from thermodynamic calculations, which indicate that the free energy available from the reaction can be as low as −20 kJ/mol (Thauer et al., 1977).

Biochemistry of Long-Chain Fatty Acid Metabolism

Syntrophic degradation of long-chain fatty acids probably involves several rounds of β-oxidation with the concomitant release of electrons as H_2 via a reversed electron transport, analogous to the pathway described for syntrophic butyrate degradation above.

Biochemistry of Crotonate Metabolism

Members of the family Syntrophomonadaceae are capable of growing in pure culture, without a syntrophic partner, when grown on crotonate (Beaty and McInerney, 1987; McInerney and Wofford, 1992). The use of crotonate as an energy source bypasses an unfavorable step, the oxidation of butyryl-CoA to crotonyl-CoA (Fig. 2, step 2) in the butyrate degradation pathway. Therefore, the bacterium generates ATP without the dependence upon interspecies electron transfer because crotonate is metabolized by a disproportionation mechanism wherein part of the substrate is oxidized to acetate and the remainder is reduced to butyrate or longer chain fatty acids (Beaty and McInerney, 1987;

McInerney and Wofford, 1992). In addition, it was reported that *Syntrophomonas wolfei* contains a *c*-type cytochrome, which may make it possible for the organism to produce ATP not only by substrate-level phosphorylation, but also through the use of an electron transport-linked reduction of crotonate (McInerney and Wofford, 1992). Localization experiments revealed that the *c*-type cytochrome is situated at the membrane and is reduced by H_2 (M. J. McInerney and N. Q. Wofford, unpublished data).

The pathway of crotonate degradation was elucidated in *S. wolfei* (McInerney and Wofford, 1992). For each crotonate that is oxidized to two acetates, another crotonate is reduced to butyrate. In the first step of the oxidation of crotonate, crotonate is activated to crotonyl-CoA by a CoA transferase. The activity of this enzyme was not present in cell-free extracts prepared from butyrate-grown cells. This suggests that the crotonate CoA transferase has an altered substrate specificity or is a new enzyme that is synthesized when crotonate is present as substrate. This is followed by the oxidation of crotonyl-CoA to acetate according to the steps 3–5 of the butyrate degradation pathway (Fig. 2). This oxidation generates one pair of electrons on NADH and one ATP. For each crotonate thus oxidized, a second crotonate is reduced to butyrate. The second crotonate is converted to crotonyl-CoA as described above, and this crotonyl-CoA is reduced by the reverse of steps 2 and 1 (Fig. 2), with $FADH_2$ the electron donor. This reductive branch does not generate ATP directly, but electrons from NADH generated from the oxidative branch (steps 3–5) are passed down to $FADH_2$ for use in reducing crotonyl-CoA, and this electron transfer can be coupled to the synthesis of a fraction of an ATP.

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CHAPTER 2.1

The Cyanobacteria—Isolation, Purification and Identification

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The oxygenic photosynthetic bacteria comprise two groups, the cyanobacteria and the prochlorophytes, that are distinguished by their photosynthetic pigment composition. The cyanobacteria contain chlorophyll *a* and phycobiliproteins as their primary photosynthetic pigments (see The Cyanobacteria—Ecology, Physiology and Molecular Genetics in this Volume) while the prochlorophytes contain chlorophylls *a* and *b* and lack phycobiliproteins (see The Genus Prochlorococcus in this Volume).

The cyanobacteria are morphologically and developmentally one of the most diverse groups of prokaryotes. They range from simple unicellular forms that reproduce by binary fission to complex filamentous forms that possess a variety of highly differentiated cell types. Some of the filamentous forms are capable of true branching, and some are even truly multicellular, as the result of cellular differentiation and functional specialization between vegetative cells and heterocysts, the sites of oxygenic photosynthesis and dinitrogen fixation, respectively.

Traditionally this group of phototrophic prokaryotes has been classified as a group of algae under the aegis of the Botanical Code (Stafleu et al., 1972). Phycologists have developed a system of classification for these organisms based on their morphological, developmental, and ecological characters, as determined not on pure cultures but on natural samples. This system, which contains about 150 genera and 1000 species, has proven to be successful for the classification of cyanophytes in natural material but is inadequate in many instances for the classification of cyanobacteria maintained in axenic culture.

During the last 25 years, following the unequivocal demonstration of the prokaryotic nature of these organisms, a number of bacteriologists have become interested in and have applied traditional microbiological techniques to the study of cyanobacteria. As a result of these studies there are currently several hundred isolates of cyanobacteria maintained in pure culture. These isolates include representatives of many, but certainly not all, of the major groups of cyanobacteria described in the botanical literature.

The classification of cultured cyanobacteria has been problematic. It was hoped that phenotypic and genotypic characters, made accessible by the availability of pure cultures, would facilitate the classification of the group. However, until recently, this, in large part, had not proven to be the case. As in the traditional botanical taxonomic treatments, morphological and developmental features form the bases for the description of taxa at the level of genera and above (Castenholz, 1989a, 1989b, 1989c; Rippka, 1988b; Waterbury, 1989). Exceptions occur among some unicellular cyanobacteria that lack adequate morphological and developmental complexity to permit genera to be defined by these criteria alone (Waterbury and Rippka, 1989). However, because of the importance of structural and developmental characters for the classification of both field and cultured material, it will be possible for the system of classification currently being developed for the cyanobacteria, under the aegis of the Bacteriological Code (Lapage, 1975) to represent, to a large degree, a logical extension and refinement of the classical botanical system.

Major Groups of Cyanobacteria

The cyanobacteria studied in pure culture are currently placed in five orders (Castenholz, 1989a, 1989b, 1989c; Waterbury, 1989; Waterbury and Rippka, 1989) described below that correspond closely to the five groups (Sections) used by Stanier and his collaborators (Rippka, 1988b; Rippka et al., 1979, 1981b).

Chroococcales

Members of the order Chroococcales are unicellular cyanobacteria that reproduce by binary fission or budding. Division occurs in one, two, or three planes at right angles to one another or in irregular planes. Cells can range in size from 0.5–30 μm and can occur as single cocci and rods or as cell aggregates. The form and size of cell

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This chapter was taken unchanged from the second edition.

aggregates depend on the planes of division and on the presence of extracellular slime or structured sheaths that hold the cells together.

Pleurocapsales

All members of the order Pleurocapsales reproduce by multiple fission, a feature that distinguishes them from other cyanobacteria. Pleurocapsalean cyanobacteria range from unicellular forms that divide exclusively by multiple fission to forms that produce cell aggregates by vegetative binary fission. Such cell aggregates range in complexity from groups of a few cells to complex pseudofilamentous cell assemblages. Following aggregate formation, some cells in the assemblage undergo multiple fission and release unicellular structures called baeocytes. Baeocytes immediately initiate growth leading to the next vegetative cell cycle.

Oscillatoriales

The order Oscillatoriales includes all the undifferentiated filamentous cyanobacteria. Cell division occurs by binary fission in one plane at right angles to the long axis of the trichomes. Reproduction occurs by trichome fragmentation or by the production of undifferentiated hormogonia released from the ends of trichomes. Although cell size and shape and trichome length vary widely among members of the order, within individual organisms, cell diameter and cell shape are quite constant. Cell diameters vary from 0.5–100 µm, and cell dimensions range from being much longer than wide (rod-shaped) to being much wider than long (disk-shaped).

Nostocales

The order Nostocales includes filamentous cyanobacteria that are capable of cell differentiation and that divide by binary fission in one plane at right angles to the long axis of the trichomes. Differentiation may result in the production of several types of specialized cells: 1) heterocysts; 2) akinetes; 3) specialized reproductive trichomes (hormogonia) whose cells are morphologically distinguishable from vegetative cells; and 4) tapered trichomes, formed usually in response to a nitrogen gradient caused by terminally located heterocysts.

Stigonematales

Members of the order Stigonematales are filamentous cyanobacteria capable of the same degree of cellular differentiation as members of the Nostocales, but in addition they are able to divide by binary fission in multiple planes. The resulting thalli may display true branching and possess both uniseriate and multiseriate trichomes.

Phylogeny

Until the advent of molecular sequencing it was not possible to quantitatively determine phylogenetic relationships among prokaryotes. Woese and his colleagues have revolutionized the study of prokaryotic phylogeny using ribosomal RNA sequence analyses. Using both partial and complete 16S rRNA sequences, Woese has divided the prokaryotes into two major subgroups, the archaebacteria and the eubacteria (Woese, 1987). The cyanobacteria, as well as the chloroplasts of higher plants and the prochlorophytes (Turner et al., 1988) all fall within a common lineage that comprises one of 10 major eubacterial taxa (Woese, 1987). At the time of Woese's review, the cyanobacteria were circumscribed by eight rRNA sequences from isolates representing only a minor portion of cyanobacterial diversity (Bonen et al., 1979).

A more detailed study by Giovannoni et al. (1988) analyzed the 16S rRNAs of 29 strains of cyanobacteria, including key reference strains from each of the five orders. This study permitted its authors to delineate the major phylogenetic patterns of the cyanobacteria currently available in pure culture. They found that the rRNA sequence diversity within the cyanobacteria is considerably less than the diversity that separates other major eubacterial taxa, indicating that relatively close phylogenetic relationships underlie the extensive morphological diversity that occurs within the cyanobacteria. In addition, many of the cyanobacterial lineages have similar branching depths (Fig. 1), indicating that the modern groups arose from an expansive radiation. The combination of relatively small sequence diversity and the fanlike radiation of the lineages makes it impossible at present to determine the precise branching orders of many of the cyanobacterial groups. Members of the Chroococcales and the Oscillatoriales are dispersed throughout the phylogenetic tree, indicating that these two orders, as presently constituted, do not represent coherent evolutionary lineages (Fig. 1). Members of the Pleurocapsales fall within a single lineage indicating that reproduction by multiple fission is of monophyletic origin (Fig. 1). Members of the Nostocales and Stigonematales fall within a single coherent lineage, suggesting that the ordinal separation of these two groups may be unwarranted (Fig. 2).

Physiological Properties Relating to Habitat Diversity

Cyanobacteria possess properties that allow them to successfully inhabit and often dominate a wide variety of illuminated habitats. Their dominant mode of nutrition is photoautotrophy,
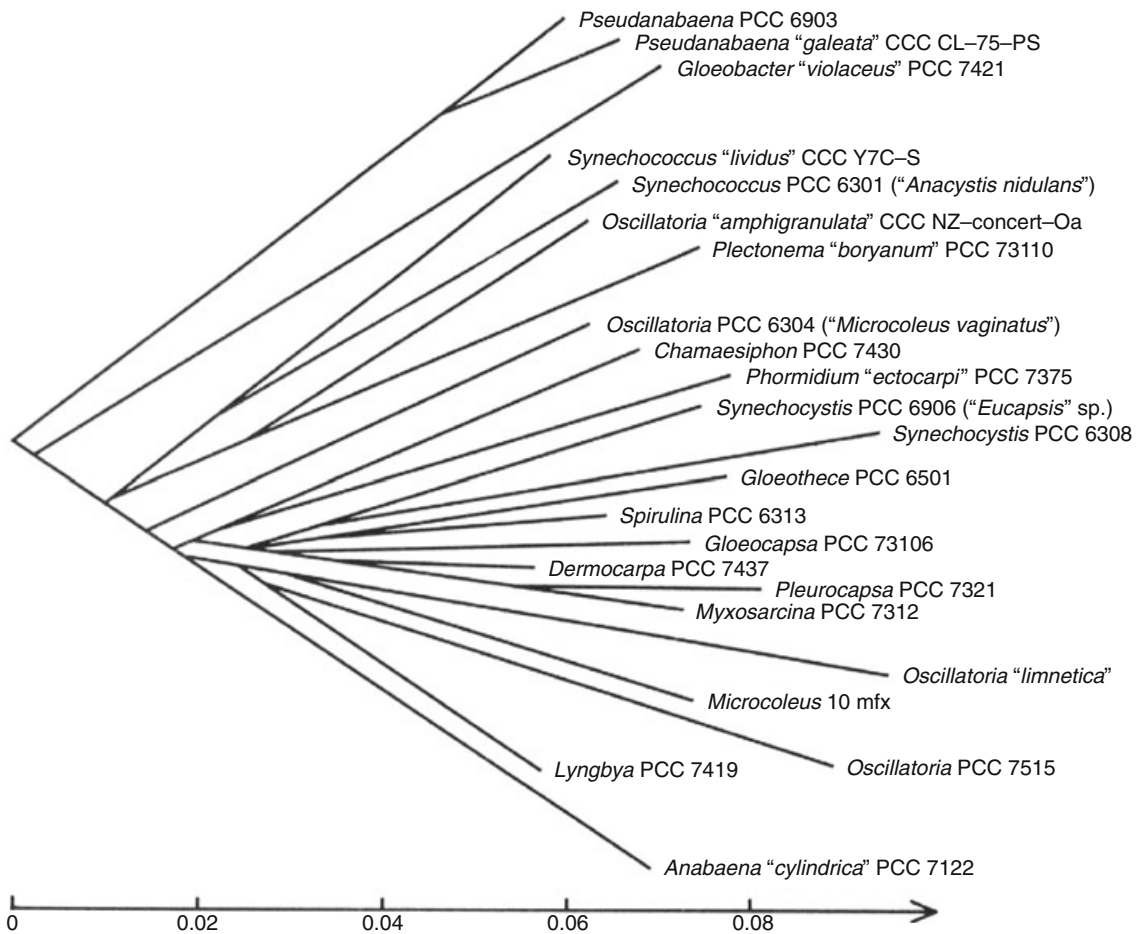
Fig. 1. Rooted-tree topology illustrating evolutionary relationships among 16S rRNAs from cyanobacteria. Evolutionary distances are proportional to the horizontal component of segment length in this representation. *A. tumefaciens, B. subtilis,* and *P. testosteroni* 16S rRNA sequences were used to locate the root. Only one heterocystous cyanobacterium, *Anabaena* sp. strain PCC 7122, is included in this tree. The scale is in units of fixed point mutations per sequence position. (From Giovannoni et al., 1988.)
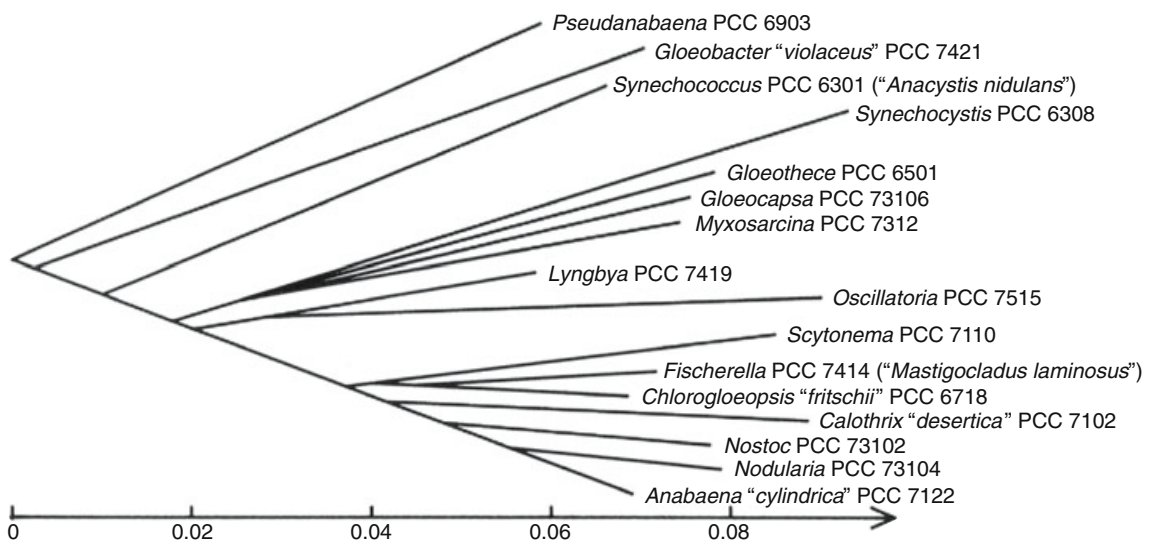
Fig. 2. Rooted-tree topology illustrating evolutionary relationships among 16S rRNAs from heterocystous cyanobacteria. See the caption of Fig. 1 for explanation. (From Giovannoni et al., 1988.)

using a photosynthetic apparatus that carries out oxygenic plantlike photosynthesis with chlorophyll *a* as the primary photosynthetic pigment and phycobiliproteins as auxilliary light-harvesting pigments (Bryant, 1986; van Liere and Walsby, 1982; see also The Cyano bacteria—Ecology, Physiology and Molecular Genetics in this Volume). Some cyanobacteria are capable of switching from oxygenic photosynthesis to anoxygenic bacterial-type photosynthesis when they occur in environments where hydrogen sulfide is present at relatively high concentrations (3 mM) (Cohen et al., 1986; Padan and Cohen, 1982).

Cyanobacteria can exploit niches varying from very dimly lit caves to salt-marsh algal mats exposed to full sunlight (Wyman and Fay, 1987). Adaptations to light include the ability to alter their photosynthetic apparatus in response to light quantity and quality by changing the surface area of their photosynthetic thylakoids, and by changing the size and phycobiliprotein composition of their phycobilisomes (Cohen-Bazire and Bryant, 1982; Glazer, 1981; Kana and Glibert, 1987a, 1987b). In addition, some of the phycoerythrin-containing cyanobacteria can adapt to changes in light quality by complementary chromatic adaptation, a process in which phycobiliprotein synthesis is under light-wavelength control (Tandeau de Marsac, 1977, 1983). Cyanobacteria also protect themselves from growth-inhibiting light intensities with a variety of pigments that adsorb harmful radiation (e.g., carotenoids and xanthophylls) and possibly through structures that scatter light (e.g., gas vacuoles and calcified sheaths) (van Liere and Walsby, 1982). Finally, some cyanobacteria have the ability to position themselves in a light field through buoyancy regulation or active motility. Cyanobacteria derive buoyancy from the possession of gas vesicles and are thought to regulate their position in a water column through the use of one or more of three mechanisms: 1) regulation of gas vesicle formation; 2) gas vesicle collapse resulting from high cell turgor pressure; and 3) changes in cell density caused by the temporal cycling of the synthesis and degradation of carbohydrate reserves (van Liere and Walsby, 1982; Walsby, 1978, 1987). Gliding motility coupled with phototaxis permits many filamentous cyanobacteria to move to areas of optimal light conditions (Castenholz, 1982). In microbial mats, for example, filamentous cyanobacteria can migrate to different levels within the mat in response to light intensity.

Cyanobacteria are predominantly photoautotrophic although some are capable of heterotrophy when grown under laboratory conditions. The ability to grow photoheterotrophically using simple organic compounds as sole carbon sources and light as a source of energy is relatively common among cultured cyanobacteria, whereas the ability to grow chemoheterotrophically in the dark using an organic compound as a sole source of carbon and energy is more restricted (Rippka et al., 1979). The significance of photoheterotrophy and chemoheterotrophy to cyanobacteria in natural environments is still unresolved (Smith, 1982). However, it is unlikely that either process will be found to be important to the metabolism of free-living cyanobacteria, since the range of substrates is limited to a restricted number of simple sugars that can be metabolized via the oxidative pentose-phosphate cycle, and the concentrations of these compounds are usually too low in nature to contribute significantly to cyanobacterial carbon metabolism.

Cyanobacteria are also capable of mixotrophy, a process in which a variety of organic compounds, such as amino acids, that cannot serve as sole carbon sources, are assimilated as a supplement to autotrophic CO_2 fixation. This use of organic compounds is potentially more important than either photo- or chemoheterotrophy to the metabolism of free-living cyanobacteria in nature, but it is poorly documented at present.

The ability of many cyanobacteria to fix dinitrogen permits them to exploit habitats low in combined nitrogen. One of the key physiological properties of nitrogenase, the enzyme system responsible for dinitrogen fixation, is its sensitivity to oxygen inactivation and repression. This presents an acute problem for nitrogen-fixing cyanobacteria that not only live in oxygenated environments but also produce oxygen during photosynthesis. Some filamentous cyanobacteria have solved this problem by spatially separating the processes of oxygenic photosynthesis and dinitrogen fixation through the production of highly differentiated cells, known as heterocysts, that are specialized sites of dinitrogen fixation (Bothe, 1982). Heterocysts lack the ability to carry out oxygenic photosynthesis. There are also a growing number of nonheterocystous, filamentous (Pearson et al., 1979; Stahl and Krumbein, 1985) and unicellular (Gallon et al., 1974; Huang and Chow, 1986, 1988; Mitsui et al., 1987) cyanobacteria that are capable of fixing dinitrogen by temporally separating these processes. In these organisms, photosynthesis occurs during the light period, while dinitrogen fixation occurs during the dark at the expense of carbohydrate reserves (glycogen) built up during the photosynthetic period.

Cyanobacteria are found in environments with quite different temperature ranges. Most cyanobacteria are mesophilic and live in environments where temperature may range from freezing to 40°C. They typically have growth optima between 20 and 35°C and maximum tempera-

tures permitting growth below 45°C. Cyanobacteria isolated from the open oceans, where temperature ranges are more moderate, often have temperature maxima near 30°C (Waterbury et al., 1986). One tropical, marine, unicellular, nitrogen-fixing cyanobacterium has a temperature range permitting growth only between 26°C and 32°C, which is one of the narrowest temperature ranges known for a free-living mesophilic prokaryote.

Cyanobacteria are also commonly found in more extreme environments. In Antarctica, they are present as cryptoendoliths in rocks in the cold dry deserts and in the plankton and microbial mats of lakes. With the exception of one strain of *Chroococcidiopsis* sp. that is a psychrophile, the cyanobacterial isolates from the Antarctic desert rocks are mesophiles, with temperature optima near 35°C (I. Friedmann, personal communication). On the other hand, many of the cyanobacteria isolated from Antarctic lakes are psychrophiles, having maximum temperatures permitting growth of 20°C (Seaburg, 1981).

Cyanobacteria, including representatives of each of the five orders, are conspicuous inhabitants of hot springs where they occur at temperatures up to 74°C. Thermophilic strains of cyanobacteria have growth optima above 45°C and often fail to grow, but can survive at room temperature (Castenholz, 1981).

Cyanobacteria occur in habitats of widely differing salinity. Freshwater habitats contain diverse and often prominent populations of cyanobacteria. In marine habitats, cyanobacterial isolates can be divided into two categories based on their major ionic requirements for growth. Some are halotolerant and grow equally well on a medium with either a seawater or freshwater base. Others have obligate requirements for concentrations of sodium, magnesium, calcium, and chloride that reflect the chemistry of seawater. These requirements are not met by supplementing freshwater media with sodium chloride alone (Waterbury et al., 1986). Many cyanobacteria isolated from soils are tolerant of salt concentrations in excess of 1 M NaCl, whereas marine isolates usually fail to grow at this concentration, probably because the salinity of seawater has been relatively constant for long periods.

The occurrence of cyanobacteria in hypersaline environments is well documented in the classical descriptive literature (Hof and Frémy, 1933) but only a very restricted group is truly halophilic. Individual strains of *Aphanothece halophytica* isolated from a salt evaporation pond (Yopp et al., 1978), from Great Salt Lake, Utah (Brock, 1976), and the Solar Lake, Sinai, Israel (Cohen, 1975) each have major ionic requirements for growth that reflect the chemistry of their individual habitats. The isolates from the salt evaporation pond and from Great Salt Lake are truly halophilic. They grow optimally in approximately 2 M NaCl and have minimum NaCl requirements for growth of 0.7 M and 1.0 M NaCl, respectively.

Cyanobacteria are most prominent in habitats with neutral to alkaline pH. They are excluded from highly acidic environments but are characteristic inhabitants of acidic hot springs and peat bogs where the pH is above 5 (Castenholz, 1981, 1988b). To date, isolates from acidic hot springs and peat bogs grow optimally at neutral pH, indicating that they are mildly acid-tolerant rather than acidophilic (Rippka et al., 1981a). A restricted number of cyanobacteria are characteristic of highly alkaline habitats; for example, *Spirulina platensis*, a dominant cyanobacterium in highly alkaline lakes, has a pH optima for growth between 8 and 11 (Ciferri, 1982).

Cyanobacteria are often found under conditions of extreme desiccation in habitats ranging from soils to both tropical and Antarctic deserts (Fogg et al., 1973). Viability can be maintained for extended periods under desiccated conditions but active growth is limited to wet seasons (de Winder et al., 1989).

Although cyanobacteria are present, and in many instances, conspicuous, under extreme conditions of temperature, salinity, pH, and desiccation, cultures isolated from these habitats often possess optima for growth that are more moderate. Thus, many cyanobacteria seem to be able to tolerate extreme environments but relatively few are obligate extremophiles.

In addition to the diverse habitats occupied by free-living forms, cyanobacteria also occur in symbiosis with a wide variety of eukaryotes (see Cyanobacterial-Plant Symbioses in Volume 1). The ability of cyanobacteria to fix dinitrogen usually plays an important role in these associations.

Isolation and Purification

There have been numerous reviews detailing procedures for the isolation and purification of cyanobacteria. General treatments include Castenholz (1988a), Castenholz and Waterbury (1989), and Rippka (1988a). Articles concerning specific groups of cyanobacteria include Castenholz (1988b) for thermophiles, Wolk (1988) for filamentous nitrogen fixers, and Mitsui and Cao (1988), Waterbury et al. 1986, and Waterbury and Willey (1988) for marine cyanobacteria. In addition, the chapters on cyanobacteria included in this Volume (Starr et al., 1981) contain methods and media that are still timely. The discussion here is a synopsis of the literature cited above.

Collection and Treatment of Samples

Many cyanobacteria are conspicuous in nature, a feature that greatly facilitates sample collection. They are conspicuous as major components of microbial mats in both freshwater and marine marshes, in desert microbial crusts, on moist rocks in terrestrial habitats, and in the marine intertidal zone and as epiphytic colonies on terrestrial and freshwater plants and marine macroalgae. They are often less conspicuous in aquatic environments but characteristic species of planktonic cyanobacteria form dense blooms in both freshwater and marine environments (Walsby, 1981). Even in aquatic environments where blooms are not evident, small unicellular cyanobacteria are often abundant and important components of the microbial food webs in freshwater lakes (Caron et al., 1985; Pick and Caron, 1987) and in the open ocean (Waterbury et al., 1986).

The first step in isolating cyanobacteria should be a careful description of the sampling site. In addition to the site location and description, parameters such as light intensity, temperature, pH, and salinity, should be recorded. This information is not only useful for describing the natural habitat of the cyanobacteria to be isolated but can be critically important when designing culture media and conditions of incubation.

Samples for enrichment of cyanobacteria that are not growing submerged in water can be sampled by removing rock chips, microbial tufts, etc., and placing them in small containers. The collected samples should not be immersed in water but should be kept moist by the addition of wetted paper to avoid growth of contaminating microorganisms. Precaution should be taken to avoid contamination in collecting and storing water samples from which cyanobacteria are to be cultured. In extreme cases, for example, to isolate cyanobacteria from very oligotrophic waters, it is necessary to rigorously clean water samplers, storage containers, and culture vessels with acid followed by repeated washings with ultrapure deionized water. Samples of natural populations should be transported to the laboratory for processing as quickly as possible, taking care to avoid exposing them to extremes of temperature or light.

Prior to culturing, samples should be examined carefully and if possible photographed using light microscopy to determine and document the identity and relative abundance of the cyanobacteria present and to assess the extent of contamination by undesirable microorganisms. The identification of cyanobacteria in field samples is best achieved using the botanical descriptive literature. The treatise of Geitler (1932) or later treatises that follow the "Geitlerian School" have proven to be the most useful (Anagnostidis and Komárek, 1985, 1988; Bourrelly, 1985; Desikachary, 1959; Komárek and Anagnostidis, 1986).

Portions of nonaqueous natural samples in which the cyanobacteria are conspicuous should be preserved (usually by drying) and deposited in a recognized herbarium to satisfy the botanical requirement for type material. In practice, good photomicrographs of the natural material will provide excellent supplementary documentation of the type material and will aid in correlating the identity of the cyanobacteria in the natural material with the identity of cultures isolated from the sample.

Except in rare instances where the natural sample appears to be monospecific, it will be necessary to physically manipulate the sample both to break up compact clumps of organisms and to separate the cyanobacterium of particular interest from other cyanobacteria and contaminating microorganisms. A good method of dispersal in samples that contain unicellular forms is to gently grind the natural material, wetted with sterile medium, between two sterile microscope slides. Samples containing filamentous forms can be dispersed using sterile dissecting needles to tease apart clumps or tufts of natural material that have been placed in drops of sterile medium in the bottom of petri dishes. This process is facilitated by monitoring and making the manipulations using a dissecting microscope.

Once the samples have been dispersed it is often possible to remove some of the contaminating bacteria by washing with sterile medium. This can be accomplished for samples containing unicellular cyanobacteria by repeatedly washing the cells in a small sterile filter apparatus set up with a membrane filter with a pore size slightly smaller than the cyanobacterial cell. With the aid of a dissecting microscope, samples containing filamentous cyanobacteria can often be washed by picking individual trichomes with a platinum needle or a drawn-out Pasteur pipette. The individual trichomes are then passed through successive large drops of sterile liquid medium in the bottom of a petri dish or they can be dragged through solidified agar medium to physically remove attached contaminating organisms.

Media and Growth Conditions

Many media have been described for cyanobacteria. Included here are nine different media, some of which have proven successful for the isolation and maintenance of a wide variety of cyanobacteria and some designed for the growth of more specialized groups (Tables 1 and 2).

Since they are photoautotrophs, cyanobacteria can be grown in simple mineral media. Vitamin B_{12} is the only growth factor known and is only

Table 1. Nine recipes for cyanobacterial media.

Ingredient (amount per liter)	Medium designation								
	BG-11[a]	Z8[b]	D[c]	AO[d]	Aquil[e]	SN[f]	SNAX[g]	RC[h]	YOPP[i]
Deionized water (ml)	1000	1000	1000	1000	1000	250	250	1000	1000
Seawater (ml)						750	750		
NaCl (g)			0.008	1.0	24.36			125	116.9
$MgSO_4 \cdot 7H_2O$ (g)	0.075	0.25	0.1	0.2				3.5	10.0
$MgCl_2 \cdot 6H_2O$ (g)					11.03			10.0	10.68
KCl (g)					0.7			2.5	2.0
Na_2SO_4 (g)					4.09				
K_2SO_4 (mg)				1000					
$CaSO_4 \cdot 2H_2O$ (mg)			60						
$CaCl_2 \cdot 2H_2O$ (mg)	36			40	1000–1350				500
$NaNO_3$ (mg)	1500	467	700	2500	8.5	750	75	750	
KNO_3 (mg)			100						
$Ca(NO_3)_2 \cdot 4H_2O$ (mg)		59							1000
NH_4Cl (mg)		31					5.3		
K_2HPO_4 (mg)	30			500[j]		15	1.5	15	
KH_2PO_4 (mg)									50
Na_2HPO_4 (mg)			110						
$NaH_2PO_4 \cdot H_2O$ (mg)					0.5				
Na_2CO_3 (g)	0.02	0.02		4.03[j]		0.01	0.001	0.02	
$NaHCO_3$ (g)				13.61[j]	0.2				
KBr (mg)					10.0				
NaF (mg)					3.0				
Na_2 EDTA $\cdot 2H_2O$ (mg)	1.0		80		5	0.5	0.5		
NTA (mg)			100						
Ferric ammonium citrate (brown crystals) (mg)	6.0							3.0	
Citric acid (mg)	6.0								
$FeCl_3$ (mg)			0.3						
$FeSO_4 \cdot 7H_2O$ (mg)				10					
Ferric EDTA (acid 10% Fe) (mg)									5.0
Fe EDTA[l] (ml)		10							
Glycylglycine buffer (mg)								3.0	
Micronutrients (ml)	1.0	1.0	0.5	1.0[k]	0.5	1.0	0.1	1.0	1.0
Micronutrient mix used (see Table 2)	A5 + Co	Gaffron	D micro	Gaffron or A5 + CO	PIV	Cyano	Cyano	A5 + Co	Sheridan and Castenholz
Final pH after autoclaving	7.1	—	7.5[m]	9.4–9.8		7.8–8.2	7.8–8.2	—	7.8[n]

[a]From Allen (1968).
[b]From Kotai (1972).
[c]From Castenholz (1981).
[d]From Aiba and Ogawa (1977).
[e]From Morel et al. 1979.
[f]From Waterbury and Willey (1988).
[g]From Waterbury et al. (1986).
[h]From van Rijn and Cohen (1983).
[i]From Yopp et al. (1978).
[j]These three ingredients are autoclaved as a separate solution and added to the medium after cooling.
[k]Original medium used trace metal mixes A5 + B6 (see Rippka, 1988a). Gaffron's trace metal mix is quite similar and has been substituted for A5 + B6.
[l]Solution A, 2.8 g $FeCl_3$ in 100 ml 0.1 N HCl; solution B, 3.9 gm EDTA-disodium in 100 ml 0.1 N NaOH. Add 10.0 ml solution A and 9.5 ml solution B plus water to 1 liter.
[m]pH adjusted to 8.2 with NaOH before autoclaving.
[n]pH adjusted to 7.8 with NaOH before autoclaving.

Table 2. Six recipes for micronutrient solutions.[a]

Ingredient (amount per liter)	A5 + Co[b]	D micro[c]	Sheridan and Castenholz[d]	Cyano[e]	Gaffron[f]	PIV[g]
Deionized H_2O (ml)	1000	1000	1000	1000	1000	1000
H_2SO_4 (conc.) (ml)		0.5				
HCl (conc.) (ml)			3.0			
H_3BO_3 (g)	2.86	0.5	0.5		3.1	
$MnCl_2 \cdot 4H_2O$ (g)	1.81		2.0	1.4		0.041
$MnSO_4 \cdot 4H_2O$ (g)		3.01			2.23	
$ZnSO_4 \cdot 7H_2O$ (g)	0.22	0.5		0.22	0.287	
$ZnNO_3 \cdot 6H_2O$ (g)			0.5			
ZnCl (g)						0.005
$NaMoO_4 \cdot 2H_2O$ (g)	0.39	0.025	0.025	0.39		0.004
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (g)					0.088	
$CuSO_4 \cdot 5H_2O$ (g)	0.079	0.025				
$CuCl_2 \cdot 2H_2O$ (g)			0.025			
$Co(NO_3)_2 \cdot 6H_2O$ (g)	0.049		0.025	0.025	0.146	
$CoCl_2 \cdot 6H_2O$ (g)		0.045				0.002
$VOSO_4 \cdot 6H_2O$ (g)			0.025		0.054	
$Al_2(SO_4)_3K_2SO_4 \cdot 2H_2O$ (g)					0.474	
$NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$ (g)					0.198	
$Cd(NO_3)_2 \cdot 4H_2O$ (g)					0.154	
$Cr(NO_3)_3 \cdot 7H_2O$ (g)					0.037	
$Na_2WO_4 \cdot 2H_2O$ (g)					0.033	
KBr (g)					0.119	
KI (g)					0.083	
$FeCl_3 \cdot 6H_2O$ (g)						0.097
Disodium EDTA (g)						0.75[h]
Citric acid $\cdot$ H_2O (g)				6.25		
Ferric ammonium citrate (brown crystals) (g)				6.00		
Amount added per liter of medium	1.0 ml	1.0 ml	1.0 ml	0.1–1.0 ml	0.1 ml	0.5 ml

[a]It is usually best to dissolve each compound separately and then add dissolved components together and bring to 1 liter.
[b]From Rippka et al. 1979.
[c]From Castenholz (1981).
[d]From Waterbury and Stanier (1981).
[e]From Waterbury et al. 1986.
[f]From Hughes et al. 1958.
[g]From Starr (1978).
[h]The disodium EDTA should be added first.

required by a few marine isolates (Waterbury and Stanier, 1978). Media should be designed to mimic the chemical composition of the environment from which cyanobacteria are to be isolated. In addition, they must be supplemented with essential nutrients needed to support cell growth, including sources of nitrogen, phosphorus, trace elements, etc. When cyanobacteria are being isolated from extreme environments, it is prudent to use two different media: one having the characteristics of the extreme environment and one in which the extreme variable(s) is moderated. Nutrient levels in growth and maintenance media are usually high to support large cell yields. It is often advantageous to dramatically lower the nutrient levels in media that are used for enrichment and isolation. For example, compare the high nutrient levels in medium BG-11 with those in Aquil (Table 1).

To date, several hundred cyanobacteria, including representatives from most of the major groups, have been isolated and purified using standard microbiological techniques. Despite this success there still are many cyanobacteria that have not yet been cultured. Of these, cyanobacteria characteristically found in the oligotrophic waters of the oceans and freshwater lakes have been particularly problematic.

It is likely that our inability to culture certain cyanobacteria is not because we fail to add something to the medium that the organisms require, but rather because we inadvertently add constituents that are toxic. Toxicity may derive from contaminants left on the surface of inadequately cleaned samplers and culture vessels, from contaminants present in either the chemicals or water used to make culture media, or from the intentional addition of necessary compounds to culture media in quantities high enough to become toxic.

The addition of copper to cyanobacterial media is an interesting example. Copper is a nearly universal component of trace element solutions used to supplement culture media for a wide variety of microorganisms, including cyanobacteria (Table 2). However, copper has

also been shown to be extremely toxic. Rueter et al. (1979) examined the effects of copper on *Trichodesmium thiebautii*, a prominent and, at that time, unculturable oceanic cyanobacterium. They found that the addition of 10^{-8} M copper caused a marked decrease in the rate of CO_2 fixation by natural populations of *T. thiebautii*. Waterbury and Stanier (1978) used a marine medium (MN) to isolate and culture a wide variety of intertidal marine cyanobacteria. Medium MN had a natural seawater base and used nutrient additions and trace metals from medium BG-11 (Tables 1 and 2). It proved quite successful for the isolation of coastal marine cyanobacteria but would not support the growth of open-ocean strains of marine *Synechococcus* spp. (Waterbury et al., 1979, 1986). The fact that medium MN contained 3.2×10^{-7} M copper, more than was necessary to inhibit photosynthesis in *Trichodesmium* sp., led us to design medium SN, whose principal difference from medium MN was the omission of copper from its trace metal solution (Table 2). Medium SN and its more dilute form, SNAX, have been used successfully for the maintenance and isolation of many oceanic and coastal marine cyanobacteria.

Ohki et al. (1986) succeeded in culturing *Trichodesmium thiebautii* and *T. erythraeum* using the artificial seawater medium Aquil (Morel et al., 1979; see also Table 1) in which the phosphate was reduced to 0.5 µg/l and copper was deleted from the trace element solution (PIV) (Table 2). Aquil was designed to minimize heavy metal contamination by passing its major nutrients through Chelex 100 columns (Bio-Rad Laboratories, Richmond, CA). Two factors seem to have contributed to the successful culturing of *Trichodesmium* spp.: the removal of heavy metals from the nutrients added to Aquil and the omission of copper as a trace element.

Copper may also have played a role in the culturing of freshwater planktonic cyanobacteria. Medium Z8, originally described by Kotai (1972), has been used by Skulberg and his colleagues (Skulberg, 1983) to isolate and maintain a large collection of freshwater planktonic cyanobacteria. Skulberg used Gaffron's trace element solution as described by Hughes et al. (1958). In the paper by Hughes et al. (1958), Gaffron's trace element solution did not contain copper, but by 1960 (Zehnder and Gorham, 1960) copper had been added to Gaffron's trace metal solution.

These examples suggest that it may be beneficial to omit copper from all trace element solutions used to grow cyanobacteria. If cyanobacteria have a copper requirement for growth, it can probably be met by traces present as contaminants in the water and the chemicals used to make the medium.

The quality of water and the purity of chemicals used to make media are critically important

for the successful cultivation of cyanobacteria. Freshwater media and nutrient stock solutions should be made with either double-distilled water or, preferably, with single-distilled water which has been subject to ion exchange and to activated charcoal columns such as the Millipore-Q water purifier. Marine cyanobacteria usually do best in media made with natural seawater. Seawater should be filtered through glass fiber filters at the time of collection and may be stored for extended periods prior to use in carefully cleaned plastic carboys. Chemicals used to prepare both basal media and nutrient solutions should be of the highest quality. In situations where heavy metal contamination is critical, for example, in culturing oligotrophic cyanobacteria such as *Trichodesmium* spp., both the basal medium and nutrient solutions can be treated with Chelex 100 (Morel et al., 1979).

Care should also be taken to assure that glass and plastic ware that are used to culture cyanobacteria are carefully cleaned. In situations where cleanliness is critical, for example, to avoid trace metal contamination, the following protocol that is a modification of one proposed by Fitzwater et al. (1982) can be used. Glass or plastic ware are soaked in Micro detergent (International Products Corporation, Trenton, NJ 08602-0118) at a final concentration of the detergent of 20 ml per l of Super-Q water (Millipore) for one week with occasional agitation. The vessels are then rinsed eight times in Super-Q water and then soaked in high-quality 0.5 N HCl for an additional week, followed by eight washes in Super-Q water.

Liquid Media

Medium BG-11 (Table 1), originally described by Hughes et al. (1958) and modified by Allen (1968), has been widely used for the isolation and maintenance of many cyanobacteria. When it is used as an enrichment medium it is beneficial to reduce the nitrate concentration by as much as a factor of 10 (Rippka, 1988a). A variant of this medium called BG-11_0 lacks sodium nitrate and is used for cyanobacteria capable of dinitrogen fixation. Sodium lost by the removal of sodium nitrate should be replaced by the addition of 1.0 g/liter NaCl to medium BG-11_0.

Medium Z8, originally described by Kotai (1972), has been used by Skulberg (1983) to isolate and maintain a large collection of freshwater planktonic cyanobacteria. The omission of copper from its trace element solution may be the key to its ability to support the growth of freshwater oligotrophic cyanobacteria.

Medium D was developed by Castenholz (1981) to isolate and maintain thermophilic cyanobacteria. Variants of this medium (Casten-

holz, 1988a) have also been used to culture a wide variety of nonthermophilic cyanobacteria.

Medium AO is a very alkaline medium used by Aiba and Ogawa (1977) for the isolation and maintenance of *Arthrospira platensis,* a filamentous cyanobacterium characteristic of alkaline lakes.

Aquil is a defined marine medium designed by Morel et al. (1979) for trace metal studies with marine phytoplankton. Ohki et al. 1986 used Aquil modified by a threefold reduction in the phosphate concentration to successfully culture *Trichodesmium* spp., an important oceanic dinitrogen-fixing cyanobacterium that had hitherto been unculturable. Detailed instructions for the preparation of Aquil are given in Morel et al. (1979).

Medium SN and its more dilute counterpart SNAX are marine media that have been used to maintain and isolate a wide variety of open-ocean unicellular cyanobacteria (Waterbury et al., 1986; Waterbury and Willey, 1988). They have replaced medium MN (Waterbury and Stanier, 1978, 1981), which had been used to culture coastal isolates but failed to support growth of the open-ocean cyanobacteria. The principal difference between them is that medium SN contains no added copper in the Cyano trace element solution (Table 2).

Medium RC is a medium used by van Rijn and Cohen (1983) to support the growth of a halotolerant strain of *Aphanothece halophytica* isolated from Solar Lake, Sinai, Israel. This strain grew optimally at salinities between 0.5 and 1 M NaCl but could tolerate salinities approaching 3 M NaCl.

The YOPP medium was designed to support the growth of an obligately halophilic strain of *Aphanothece halophytica* that was isolated from a salt evaporation pond in the southern San Franciso Bay (Yopp et al., 1978). Their strain grew optimally in approximately 2 M NaCl and had a minimum NaCl requirement for growth of 0.7 M.

Solid Media

Solid media should be prepared by separate autoclaving of the agar and mineral solutions, and subsequent mixing after they have cooled to 50°C (Allen, 1968). The agar concentration should be kept as low as possible, maintaining just enough firmness to permit streaking. Growth of cyanobacteria is often slow, requiring long incubation times. To prevent the agar plates from drying they should contain approximately 40 ml of medium per petri dish and should be incubated in clear plastic boxes (available in stores as vegetable crispers or sweater boxes).

The general protocol described has been modified as follows for more fastidious marine unicellular cyanobacteria (Waterbury et al., 1986; Waterbury and Willey, 1988): Solid media are prepared using Difco Bacto-agar that has been further purified as follows: 100 g of agar is washed by stirring with 3 liters of double-distilled water in a 4-liter beaker. After 30 min of stirring, the agar is allowed to settle, the wash water is siphoned off, and the agar is filtered onto Whatman F4 filter paper in a Büchner funnel. This procedure is repeated once more or until the filtrate is clear. The agar is then washed with 3 liters of 95% ethanol followed by a final 3-liter wash with analytical grade acetone. The agar is then dried at 50°C in glass baking dishes for 2–3 days and stored in a tightly covered container. Solid media prepared with the purified agar at a final concentration of 0.7% are sufficiently stable for streaking.

To prepare 40 agar plates of medium SN from 1 liter of medium, the following three solutions are prepared and autoclaved separately: 1) 750 ml of filtered seawater in a Teflon bottle; 2) 7.0 g super-clean agar in 200 ml of double-distilled water in a 2-liter glass flask; and 3) the mineral salts for 1 liter of medium in 50 ml of double-distilled water in a 125-ml glass flask. After autoclaving, the seawater and minerals are added to the flask of agar. Sterile sodium sulfite (2 mM final concentration) is added aseptically to the hot agar solution, which is then cooled to 50° before the plates are poured. It is critical that the surface of agar plates be dry prior to streaking.

Conditions of Incubation

Light Although cyanobacteria occur naturally under a wide range of light conditions, they usually grow best when cultured at light intensities varying from 10–75 $\mu Ein \cdot m^{-2} \cdot sec^{-1}$ supplied by warm- or cool-white fluorescent lamps. Traditionally, cyanobacteria have been cultured under conditions of constant illumination; however, some cyanobacteria, for example, nonheterocystous nitrogen fixers that temporally separate dinitrogen fixation and photosynthesis, require a light-dark cycle. We recommend that during isolation and purification, cultures be incubated in a light-dark cycle with a 14-h light period and a 10-h dark period.

Temperature The temperature of incubation during isolation and purification should be chosen on the basis of the temperature range of the natural environment where the sample was taken. During the initial stages of isolation it is prudent to incubate samples at several temperatures until the range that will support good growth is determined.

Techniques of Isolation and Purification

Isolation and purification are facilitated by the fact that cyanobacteria are prominent and visible components of many habitats. Natural samples can be collected, dispersed, and the individual cyanobacteria isolated directly. Samples in which unicellular forms predominate can be streaked on agar plates. Samples rich in gliding filamentous forms can be teased apart and small portions of inoculum placed in the center of agar plates. Whole trichomes or hormogonia will glide away from the central inoculum, and these migrating trichomes can be removed with a platinum spade by cutting out a small block of agar and transferring it to a new agar plate. In addition to the direct isolations on agar plates, secondary cultures for each sample should be established in the appropriate liquid medium. Aqueous samples that are too dilute to isolate colonies directly on agar plates should be enriched in a liquid medium. Serial dilution of aqueous samples during the enrichment process often aids in isolating cyanobacteria.

Although successive streaking or transferring from agar plate to agar plate is the standard method for purifying cyanobacteria, there are innumerable variations that can be tried, many of which are described in the reviews cited at the beginning of the section on isolation and purification.

Purification of unicellular cyanobacteria can be achieved by streaking on solid media. It is usually advisable to transfer isolated colonies picked from agar plates into liquid medium and to allow them to grow up before restreaking. Motile filamentous cyanobacteria can be purified by transferring individual trichomes or hormogonia to new agar plates and allowing them to glide away from the contaminants before transferring them to a successive plate.

Small heterotrophic bacteria are frequently nearly invisible contaminants of cyanobacterial cultures, and success at purifying cyanobacteria requires diligence. Agar plates should be examined daily with a dissecting microscope. The time-window within which it is possible to detect isolated cyanobacterial colonies and transfer them before they become overrun by heterotrophic bacterial contaminants can be quite short. Some of the rapidly gliding filamentous forms can clean themselves of contaminants within hours of inoculation.

Careful microscopic examination of stationary phase cultures, using a combination of phase contrast and epifluorescence illumination, is the most rigorous test of purity.

Maintenance of Pure Cultures

Stock Cultures

The majority of cyanobacteria currently in pure culture are best maintained on agar slants. However, some of the planktonic cyanobacteria, especially the heavily gas-vacuolated forms, and rapidly gliding filamentous forms should be maintained in liquid cultures. Nitrogen fixers should be maintained on media devoid of combined nitrogen.

The intervals between successive transfers of stock cultures are variable. Some of the more sensitive strains must be transferred every two weeks, while others on slants may last months. Stock cultures should be incubated in low light at a temperature appropriate for the individual strains. Stock cultures of cyanobacteria should never be stored in a dark refrigerator!

Long-Term Preservation

Storage in liquid nitrogen is the best method for the long-term preservation of cyanobacteria. Dimethylsulfoxide (5–10% v/v) is added as a cryoprotectant to stationary phase cultures that have been concentrated by centrifugation. The cell concentrate is dispensed into plastic cryogenic ampules and frozen slowly at $1°C/min$ until they reach $-80°C$. The slow freezing is satisfactorily accomplished by placing the ampules in a $-80°C$ freezer for one hour. The ampules are then placed in either the vapor or liquid phase of the liquid nitrogen storage container. Samples are reactivated by placing them in a $37°C$ water bath and transferring them into fresh liquid medium immediately upon thawing. Heavily gas-vacuolated cyanobacteria can be successfully frozen in liquid nitrogen if their gas vacuoles are collapsed by centrifugation prior to freezing (Rippka et al., 1981a).

The Identification of Cyanobacteria in Pure Culture

The keys and descriptions of cyanobacteria in pure culture used here are based on the treatises of Rippka et al. (1979, 1981b) and Rippka (1988b), and on the first treatment of the cyanobacteria in *Bergey's Manual of Systematic Bacteriology* (Castenholz, 1989a, 1989b, 1989c; Waterbury, 1989; Waterbury and Rippka, 1989). The descriptions of many of the genera and "groups" are provisional. Even though several hundred different cyanobacteria have been obtained in pure culture and partially characterized, there remains an enormous amount of work before a satisfactory system of classification for

this important group of photosynthetic prokaryotes is achieved. The following key may be useful, however.

Key to the Orders of Cyanobacteria

A. Unicellular or nonfilamentous aggregates of cells held together by extracellular slime or sheath layers .... B
A. Filamentous: Range of morphology from simple trichomes to highly differentiated branching C
B. Reproduction by binary fission in one, two, or three planes, or by budding...................... Order Chroococcales
B. Cell division by multiple fission or by a combination of multiple fission and binary fission ....
.... Order Pleurocapsales
C. Cell division by binary fission in one plane at right angles to filament axis. Cell differentiation absent (terminal cells may be differentiated). Reproduction by trichome fragmentation or undifferentiated hormogonia
.... Order Oscillatoriales
C. Cell division by binary fission in one or more planes. Differentiation occurs resulting in specialized cells and structures .... D
D. Cell division in one plane at right angles to trichome axis... Order Nostocales
D. Cell division in one or more planes resulting in complex... Order Stigonematales

Comments on Genera and Groups of the Order Chroococcales

The order Chroococcales contains all the unicellular cyanobacteria, including those that divide by binary fission (family Chroococcaceae) and by budding (family Chamaesiphonaceae) (Fig. 3). Several of the genera shown in Fig. 3 are provisional and are more properly called "groups" (see below). The presence or absence and characteristics of cell aggregates and the number and regularity of the planes of division are the major characteristics used to delineate genera and groups of unicellular cyanobacteria in culture and to describe genera in the traditional botanical literature, as shown in the key below.

Key to the Genera and Groups of the Order Chroococcales*

A. Reproduction by binary fission .... B
A. Reproduction by repeated budding from the apical pole of the cell; cells ovoid; thylakoids present................
.... Genus *Chamaesiphon*
B. Thylakoids present.. C
B. Thylakoids absent; division in one plane; cells rod-shaped; sheath present........................ Genus *Gloeobacter*
C. Division in one plane .... D
C. Division in two or three planes .... F
D. Cell diameter of >3 μm; capable of aerobic N$_2$ fixation or nitrogenase produced anaerobically .... E
D. Cell diameter of <3 μm.............. *Synechococcus* group
E. Cells rod-shaped; sheath present.....................................
.... Genus *Gloeothece*
E. Cells rod-shaped; sheath absent ..... *Cyanothece* group
F. Cells coccoid to hemispherical, held together in aggregates by multilaminated sheath material..................
.... *Gloeocapsa* group
F. Cells coccoid, occurring singly, in pairs, or in aggregates held together by amorphous capsular material................
.... *Synechocystis* group

*Adapted from Waterbury and Rippka (1989).
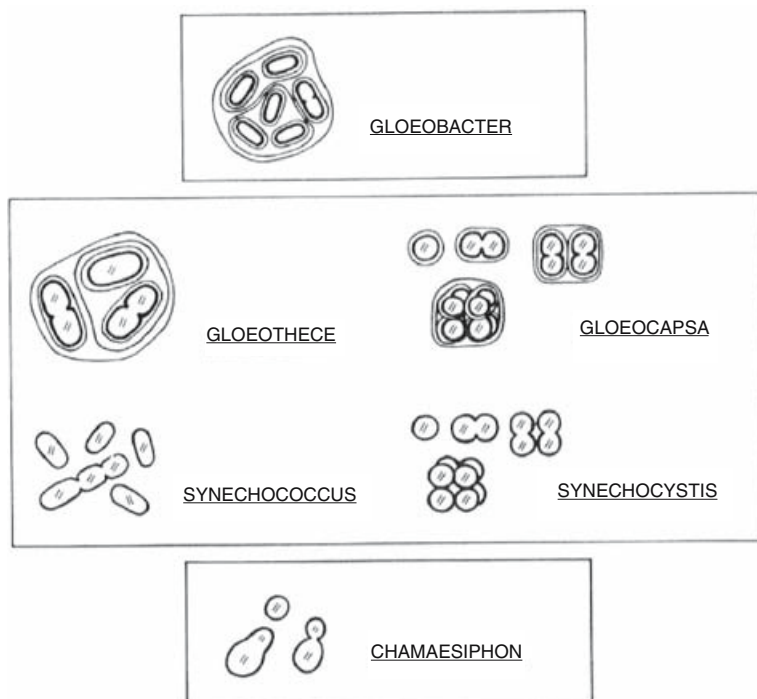
Fig. 3. Schematic presentation of the genera assigned to the order Chroococcales. The thin lines surrounding cells designate sheath material; the presence of thylakoids is indicated by the symbol //.

Cell aggregates in the Chroococcales are held together by multilaminated sheath material or by amorphous slime or capsular material (Fig. 3). The possession of extracellular sheath layers has proven to be a stable feature of many cyanobacterial groups in culture and is a primary character used in the description of several unicellular taxa including: *Gloeobacter*, *Gloeothece*, and the *Gloeocapsa* group. Other unicellular cyanobacteria, primarily some members of the *Synechocystis* group, occur in cell aggregates in nature (and, more rarely, in culture) that are held together by amorphous slime or capsular material. Slime production has proven to be an unreliable taxonomic character because its production in culture is affected by the growth phase of the cyanobacteria and the conditions under which they were grown.

In the Chroococcales, transverse binary fission occurs in one, two, or three successive planes at right angles to one another or in irregular planes. The number and regularity of the successive planes of division are stable features of individual cyanobacteria that should in principle be readily determinable from cultured material. However, in practice, it is often difficult to determine the number of successive planes of division, with the distinction between division in two or three planes being especially problematical. As a consequence, the planes of division have not been determined for many of the strains currently in pure culture, with the result that some of the loosely defined groups, particularly the *Synechocystis* group, contain some strains that divide in two successive planes and some that divide in three successive planes.

The generic description of *Gloeothece* conforms to traditional botanical usage. Members of this genus are rod-shaped cyanobacteria that divide by transverse binary fission in a single plane. The cells occur in aggregates held together by well-defined sheath layers. The cells contain intracellular photosynthetic thylakoids, a feature that distinguishes them from *Gloeobacter*. All the strains currently placed in this genus are capable of dinitrogen fixation. *Chamaesiphon* contains unicellular cyanobacteria that divide by budding (Fig. 3). This definition conforms to botanical usage except for a difference in terminology. In the botanical literature, the smaller daughter cell (bud) resulting from unequal binary fission is termed an "exospore." *Gloeobacter* is a new genus that is not described in the botanical literature and is distinguished from other cyanobacteria by the lack of intracellular photosynthetic thylakoids (Rippka et al., 1974).

The four groups included in the Chroococcales by Waterbury and Rippka (1989) reflect the provisional nature of the taxonomy of cyanobacteria based on pure cultures. The groups are divided into "clusters," each equivalent to but not given the formal status of a genus.

The *Synechococcus* group contains coccoid to rod-shaped cyanobacteria with cells smaller than 3 μm in diameter that divide by binary fission in a single plane and that lack structured sheaths. Morphologically, all members of this group appear deceptively similar. The true extent of heterogeneity within the group is apparent from the span of the GC contents of their DNAs, which range from 39–71 mol%. Waterbury and Rippka (1989) divided the *Synechococcus* group into six clusters, three marine and three freshwater. Further subdivision and arrangement of these clusters will occur before genus designations are proposed and formalized. For example, in a phylogenetic study of the cyanobacteria, Giovannoni et al. (1988) showed that two strains included in the *Synechococcus* cluster, PCC 6301 (*"Anacystis nidulans"*), a freshwater clone, and Y7C-S (*"Synechococcus lividus"*), a thermophilic clone, are on independent deep branches in the cyanobacterial phylogenetic tree (Fig. 1). The phylogenetic distance between these two strains indicates that they should be assigned to two genera.

The *Cyanothece* group contains coccoid to rod-shaped cyanobacteria with cells larger than 3 μm in diameter that divide by binary fission in one plane and that lack sheaths. This group is superficially distinguished from the *Synechococcus* group by cell size and from *Gloeothece* and *Gloeobacter* by the absence of well-defined sheath layers. Many of the strains assigned to this group by Waterbury and Rippka (1989) are either capable of dinitrogen fixation or synthesize nitrogenase under anaerobic conditions. The heterogeneity of the group is evidenced by the fact that it currently contains freshwater, marine, and halophilic isolates.

The *Gloeocapsa* group contains cyanobacteria that divide by binary fission in two or three planes at right angles to one another, resulting in cell aggregates held together by multilaminated sheath material. In the traditional botanical literature, members of this group would be placed in either *Gloeocapsa* or *Chroococcus*.

The *Synechocystis* group contains unicellular cyanobacteria that divide by binary fission in two or three planes at right angles to one another. The cells typically occur singly or in pairs in culture, but in nature and, rarely, in culture, some can also occur in aggregates held together by amorphous capsular material. Waterbury and Rippka (1989) provisionally subdivided the group into four clusters, one containing marine isolates and three containing freshwater isolates.

Some representatives of the Chroococcales are illustrated in The Phototrophic Prokaryotes in the second edition, Figs. 8 and 10.

Comments on Genera and Groups of the Order Pleurocapsales

The order Pleurocapsales contains cyanobacteria that reproduce by the formation of small spherical cells (called baeocytes) produced through multiple fission. The unicellular genera of the order divide exclusively by multiple fission. In other genera, cell aggregates are produced by binary fission, after which some or all of the cells in the aggregates undergo multiple fission and release baeocytes (Fig. 4). The small vegetative cells that are the products of multiple fission, termed "endospores" in the botanical literature, were renamed baeocytes (Greek for small cells) by Waterbury and Stanier (1978) to avoid confusion with the bacterial endospore. The ordinal and generic definitions used here and in *Bergey's Manual of Systematic Bacteriology* (Waterbury, 1989) are those proposed by Waterbury and Stanier (1978). Some of the botanical generic definitions were modified to incorporate new properties and the reinterpretation of features from the botanical descriptive literature that were documented during the study of developmental cycles in culture.

Key to the Genera and Groups of the Order Pleurocapsales*

A. Cell division solely by multiple fission .... B
A. Cell division by a combination of binary and multiple fission C
B. Baeocytes motile .... Genus *Dermocarpa*
B. Baeocytes nonmotile .... Genus *Xenococcus*
C. Baeocyte development leads to the formation of a vegetative cell that undergoes one to three binary fissions, producing a single apical cell which divides by multiple fission and releases baeocytes; the basal cell subsequently enlarges and repeats the cycle ...... Genus *Dermocarpella*
C. Baeocyte development, followed by repeated binary fission, to produce cell aggregates of varying size and complexity .... D
D. Binary fission occurs in three planes at right angles to one another, producing a cubical aggregate of cells, all of which normally undergo multiple fission .... E
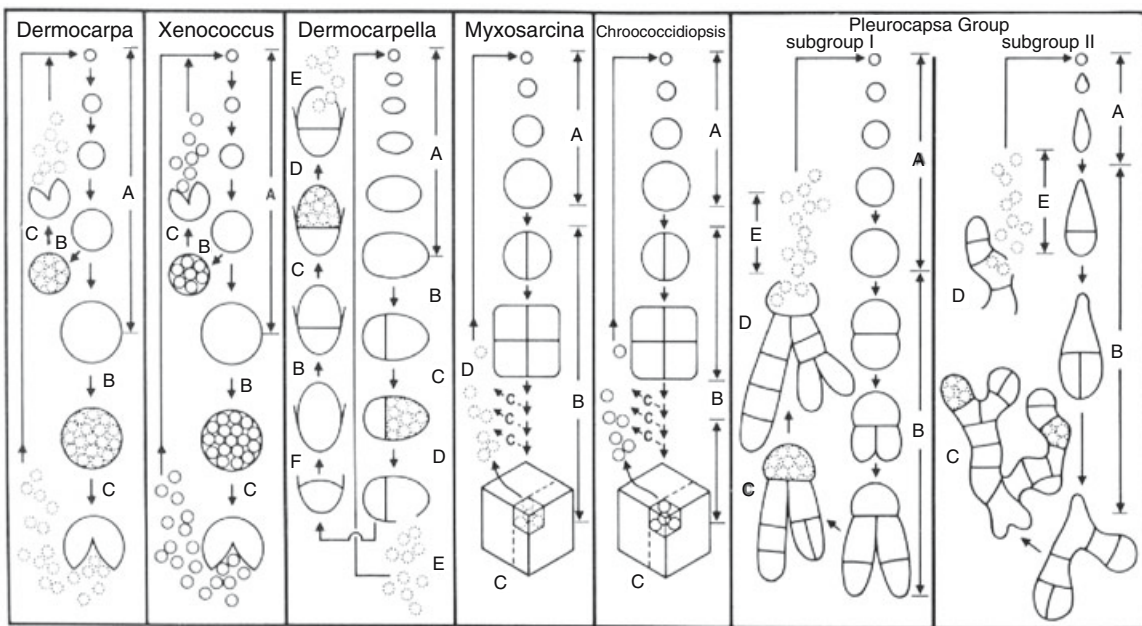
Fig. 4. Schematic presentation of genera assigned to the order Pleurocapsales. Baeocytes that are not surrounded by a fibrous (F) layer at the time of release and are, consequently, motile are symbolized by dotted circles to distinguish them from baeocytes that are surrounded by an F layer (solid circles). *Dermocarpa:* A, symmetric baeocyte enlargement; B, multiple fission, leading to baeocyte formation; C, baeocyte release followed by a brief period of baeocyte motility. *Xenococcus:* A, symmetric baeocyte enlargement; B, multiple fission leading to baeocyte formation; C, release of immotile baeocytes. *Dermocarpella:* A, asymmetric baeocyte enlargement; B, binary fission, giving rise to a small basal cell and a larger apical cell; C, multiple fission of the apical cell, leading to baeocyte formation; D, baeocyte release; E, period of baeocyte motility; F, enlargement of basal cell. *Myxosarcina:* A, symmetric baeocyte enlargement to a predetermined size; B, repeated binary fission in three regular planes; C, multiple fission of almost all the cells in the aggregate, followed by baeocyte release; D, period of baeocyte motility. *Chroococcidiopsis:* A, symmetric baeocyte enlargement to a predetermined size; B, repeated binary fissions in three regular planes; C, multiple fission of almost all the cells in the aggregate, followed by the release of immotile baeocytes. *Pleurocapsa* subgroup I: A, symmetric baeocyte enlargement; B, binary fissions in many irregular planes; C, multiple fission of some vegetative cells; D, baeocyte release; E, period of baeocyte motility. *Pleurocapsa* subgroup II: A, asymmetric baeocyte enlargement; B, binary fission in many irregular planes; C, multiple fission of some vegetative cells; D, baeocyte release; E, period of baeocyte motility. (From Waterbury and Stanier, 1978.)

D. Binary fission occurs in many different planes, to produce irregular, sometimes pseudo-filamentous aggregates of cells. Some or all of the cells in the aggregate undergo multiple fission ....*Pleurocapsa* group
E. Baeocytes motile .... Genus *Myxosarcina*
E. Baeocytes nonmotile.......... Genus *Chroococcidiopsis*

*Adapted from Waterbury (1989).

The genera *Dermocarpa* and *Xenococcus* are unicellular cyanobacteria that divide solely by multiple fission (Fig. 4). Their developmental patterns are similar, differing only with respect to baeocyte motility. The baeocytes of *Dermocarpa* are capable of gliding motility for a short period following their release from the parental cell, whereas in *Xenococcus*, the baeocytes are nonmotile. *Dermocarpella* represents the simplest pleurocapsalean cyanobacterium, whose developmental cycle incorporates division by both binary and multiple fissions. The baeocyte enlarges asymmetrically into an ovoid vegetative cell, which then undergoes binary fission to form a large apical cell and from one to three smaller basal cells. The large apical cell then undergoes multiple fission and releases motile baeocytes. Subsequently the basal cells enlarge and undergo binary fission and repeat the cycle. The developmental patterns of *Myxosarcina* and *Chroococcidiopsis* are very similar, differing only with respect to baeocyte motility (Fig. 4). The baeocyte enlarges symmetrically into a vegetative cell of fixed strain-specific size. The vegetative cell then begins to divide by binary fission in three alternating planes at right angles to one another to produce a large, approximately cubical, cell aggregate. Multiple fission, when it occurs, is massive; almost all the cells in the aggregate undergo cleavage and release baeocytes.

The *Pleurocapsa* group includes a large number of internally diverse strains. Their development involves baeocyte enlargement, followed by binary fission in irregular planes to produce cell aggregates that differ widely in size and complexity in different strains (Fig. 4). They range from small, compact masses of cells to large structures, consisting of a central mass of cells from which radiate more or less extensive pseudofilamentous outgrowths. Eventually, some cells in the aggregate undergo multiple fission and produce motile baeocytes. The baeocyte enlarges symmetrically, into a spherical vegetative cell (strains of subgroup I), or asymmetrically, into an elongated vegetative cell (strains of subgroup II), before the onset of binary fission.

A representative of the genus *Dermocarpa* and a member of the Pleurocapsa group are illustrated in The Phototrophic Prokaryotes in the second edition, Figs. 11 and 12.

Comments on Genera and Groups of the Order Oscillatoriales

The order Oscillatoriales contains filamentous cyanobacteria that divide by binary fission in a single plane (Fig. 5). Trichomes are made up solely of vegetative cells; differentiation, if it occurs, is limited to morphological changes in the terminal cells of filaments.

Phycological treatments to delineate genera of this order rely heavily on the presence or absence and the characteristics of extracellular sheath material. Although sheaths are still used as a character to define oscillatorian genera in pure culture, primary emphasis is placed on the size, shape, and arrangement of the cells within filaments (Castenholz, 1989a; Rippka, 1988b).

Key to the Genera and Groups of the Order Oscillatoriales

A. Trichomes straight or loosely coiled for a portion of their length C
A. Trichomes helically coiled .... B
B. Cross-walls between vegetative cells invisible by light microscopy.................................. Genus *Spirulina*
B. Cross-walls visible between vegetative cells .... Genus *Arthrospira*
C. Cells disk-shaped (wider then long) .... D
C. Cells isodiametric or cylindrical .... E
D. Trichomes with distinct sheath .... Genus *Lyngbya*
D. Trichomes without or with very thin sheath.................. .... Genus *Oscillatoria*
E. Cells cylindrical, several trichomes harbored within a common sheath................................... Genus *Microcoleus*
E. Trichomes not within a common sheath..................... F
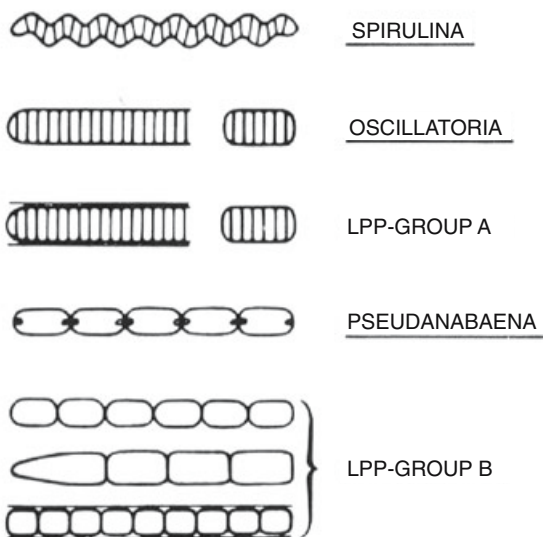
Fig. 5. Schematic presentation of the genera assigned to the order Oscillatoriales. The thin lines surrounding trichomes designate sheath material. LPP-group A consists of the genus *Lyngbya*. The polar bodies in *Pseudanabaena* are gas vacuoles.

F. Cells cylindrical, constriction occurs at cross-walls and gas vacuoles occur at cell poles..
.... Genus *Pseudanabaena*
F. Cells isodiametric or cylindrical, with or without sheaths, motile or nonmotile............................ LPP group

The genera *Spirulina* and *Arthrospira* are filamentous cyanobacteria with helically coiled trichomes (Fig. 5). In *Spirulina* the trichomes are tightly coiled, and the cross-walls between adjacent vegetative cells are not visible by light microscopy. The trichomes in *Arthrospira* are loosely coiled and the cross-walls are evident by light microscopy. Ultrastructural studies by Guglielmi and Cohen-Bazire (1982) demonstrated that the arrangement of pores in the peptidoglycan layer of the cell wall was different in *Spirulina* and *Arthrospira* but the arrangement in *Arthrospira* and members of the genus *Oscillatoria* was similar. In some botanical treatments (Bourrelly, 1985), *Arthrospira* is included within the genus *Oscillatoria*. Further study is needed to circumscribe these genera.

The genera *Oscillatoria* and *Lyngbya* (both sensu Rippka, 1988b) both include filamentous cyanobacteria with straight trichomes composed of disk-shaped cells (cells much wider than they are long) (Fig. 5). In *Oscillatoria*, the trichomes lack sheaths or are very lightly sheathed and exhibit gliding motility involving trichome rotation during translocation. The trichomes of *Lyngbya* are heavily sheathed and nonmotile. Undifferentiated hormogonia are released from the ends of the sheath and are motile for a period until new sheath material is synthesized. These definitions are more restrictive than those used by Castenholz (1989a), which also include forms with cells longer than they are wide.

The genus *Microcoleus* contains filamentous cyanobacteria with straight trichomes composed of cylindrical cells. Although this genus is not currently recognized by Rippka (1988b), because the common sheath is often not produced in culture, the common sheath is very characteristic in field material.

The genus *Pseudanabaena* contains filamentous cyanobacteria with unsheathed motile trichomes composed of cylindrical to barrel-shaped cells (Fig. 5). The cells usually contain polar gas vacuoles, and the cell walls are normally constricted at the junction between adjacent vegetative cells. They are capable of gliding motility but translocation is not accompanied by trichome rotation.

The LPP group (sensu Rippka, 1988b) contains a heterogeneous assemblage of filamentous cyanobacteria including forms that would be assigned to genera such as *Phormidium* and *Plectonema* in the botanical literature. LPP-

group A is actually *Lyngbya* (Fig. 5). A satisfactory resolution of this group awaits more detailed study.

Some representatives of the Oscillatoriales are illustrated in The Phototrophic Prokaryotes in the second edition, Figs. 9 and 13.

Comments on the Genera of the Orders Nostocales and Stigonematales

Members of the orders Nostocales and Stigonematales are distinguished from the filamentous cyanobacteria in the order Oscillatoriales by their capacity for cellular differentiation (Figs. 6 and 7). In the absence of combined nitrogen, some vegetative cells in or at the ends of trichomes develop into heterocysts. Mature heterocysts are distinguishable from vegetative cells by their thick cell walls, relatively weak pigmentation, and refractile polar granules at points of attachment to adjacent vegetative cells. When mature, a heterocyst can neither divide nor revert to a vegetative cell; it is the specific cellular site of nitrogen fixation under aerobic conditions (Wolk, 1982). Members of the Nostocales and the Stigonematales are truly multicellular as a result of cellular differentiation and functional specialization between vegetative cells and heterocysts.

Many members of these two orders also produce thick-walled resting cells known as akinetes. Mature akinetes are usually larger than vegetative cells, have a lower phycobiliprotein content, and may produce pigments that give them a brownish appearance. They also contain large amounts of reserve material (Herdman, 1987; Nichols and Adams, 1982). They are formed as cultures approach the stationary phase of growth and are usually not dependent on the nature of the nitrogen source, except in those cyanobacteria that only form akinetes adjacent to heterocysts (e.g., *Cylindrospermum*). In such cases, repression of heterocyst formation by combined nitrogen is coupled with the repression of akinete formation. Akinetes are resistant to desiccation and to cold, but not to heat. They germinate under favorable growth conditions and give rise to new filaments.

The number of planes of division is the sole character that separates the orders Nostocales and Stigonematales. In members of the Nostocales, cell division always occurs in a single plane at right angles to the long axis of the trichomes, which are consequently uniseriate and unbranched (Fig. 6). Members of the Stigonematales are capable of division in more than one plane, giving rise to true branches and both multiseriate and uniseriate trichomes (Fig. 7).

Fig. 6. Schematic presentation of the genera assigned to the order Nostocales. (a) Without developmental cycle; (b) with developmental cycle. Heavy-walled cells with polar granules represent heterocysts; heavy-walled cells that are stippled represent akinetes; thin lines surrounding trichomes designate sheath material.
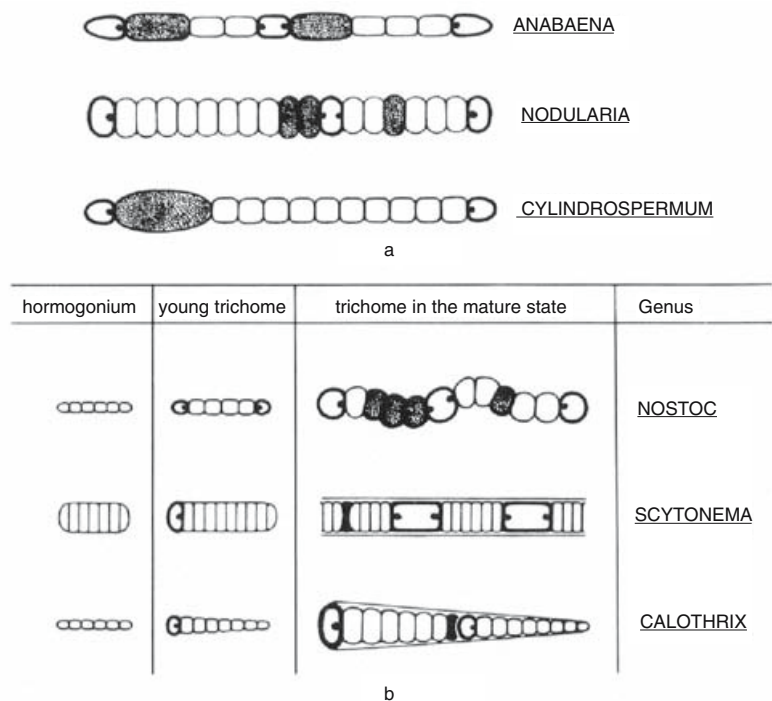
Fig. 7. Schematic presentation of two of the genera assigned to the order Stigonematales. Heavy-walled cells with polar granules represent heterocysts; heavy-walled cells that are stippled represent akinetes; thin lines surrounding groups of cells designate sheath material.
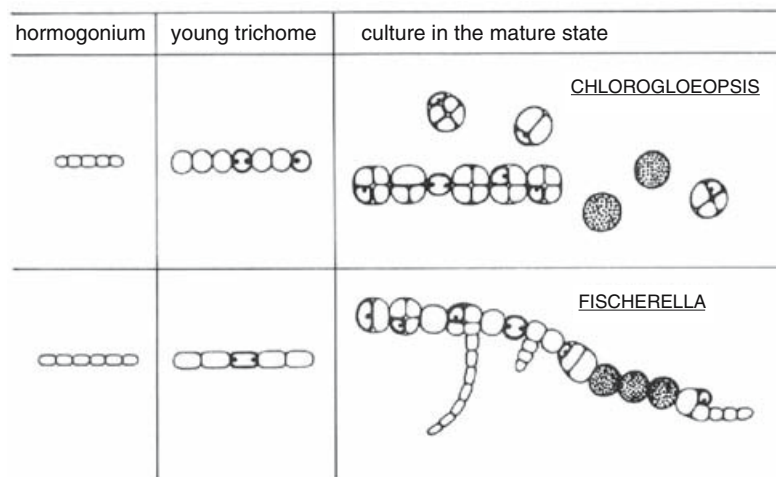
Key to the Genera of the Order Nostocales

A. Reproduction by trichome breakage (hormogonia not produced) or by germination of akinetes (if they are produced) .... B
A. Reproduction by hormogonia that are morphologically distinguishable from vegetative trichomes; reproduction may also occur by trichome breakage and akinete germination .... D
B. Heterocysts are terminal, may occur at both ends of trichomes. Akinetes (when present) are directly adjacent to heterocysts .... Genus *Cylindrospermum*
B. Heterocysts intercalary or terminal; location of akinetes (when present) variable C
C. Vegetative cells spherical, ovoid, or cylindrical.............
.... Genus *Anabaena*
C. Vegetative cells shorter than wide to disk-shaped........
.... Genus *Nodularia*

D. Heterocysts are terminal; mature trichomes taper from base near heterocyst to tip .... Genus *Calothrix*
D. Trichomes do not taper... E
E. Trichomes heavily sheathed, false branching may occur usually at site of intercalary heterocyst. Hormogonia produce heterocysts at one end of filaments only ....
.... Genus *Scytonema*
E. Trichomes not sheathed, false branching absent. Hormogonia produce heterocysts at both ends of young filaments.. Genus *Nostoc*

Nostocalean genera currently available in pure culture can be divided into two groups by their capacity to produce hormogonia: One group, the genera *Cylindrospermum*, *Nodularia*, and *Anabaena*, do not form hormogonia (Fig. 6). Reproduction in these genera is by trichome

breakage or by the germination of akinetes if they are produced. The definitions of the genera *Cylindrospermum* and *Nodularia* correspond to traditional botanical definitions: Members of the genus *Cylindrospermum* produce untapered trichomes with a terminal heterocyst. Akinetes are always produced adjacent to heterocysts. Members of the genus *Nodularia* produce untapered trichomes composed of cells that are wider than long (disk-shaped). Heterocysts in this genus are differentiated from both terminal and intercalary cells. The distinction between the genera *Anabaena* and *Nostoc* is problematic. In the botanical literature, the distinction between the two genera is made on the basis of slime production; in the genus *Nostoc*, this results in characteristic macroscopic colonies when observed in natural material. In culture, the morphology of filaments in the two genera is similar; there, their distinction rests on the production of hormogonia by *Nostoc* and their absence in *Anabaena*, a distinction that may be difficult to make because some strains of *Nostoc* only produce hormogonia erratically (Lachance, 1981; Rippka, 1988b).

Members of the genera *Scytonema* and *Calothrix* share with *Nostoc* the ability to form differentiated hormogonia (Fig. 6). However, the pattern of development of hormogonia into mature filaments differs in each genus. The hormogonia in all three genera are initially devoid of heterocysts. In *Nostoc,* the hormogonia form two heterocysts, one at each end of the filament. Subsequent growth gives rise to mature trichomes in which intercalary heterocysts are also produced. In *Scytonema* and *Calothrix*, heterocyst formation in the developing hormogonia is restricted to the terminal position at only one end of the cellular chain.

In *Scytonema*, growth and elongation of hormogonia give rise to heavily sheathed trichomes of constant width, in which heterocysts are predominantly intercalary. In *Calothrix*, hormogonia that are composed of cells of constant width give rise to tapered trichomes. The direction of tapering that is characteristic of *Calothrix* is determined by the location of the first heterocyst produced. Members of the genus *Calothrix* (sensu Rippka, 1988b) are often included in a number of traditional genera such as *Gloeotrichia*, *Rivularia*, *Isactis*, and *Dichothrix*.

Key to the Genera of the Order Stigonematales

A1. Cells of mature trichome are spherical and divide in multiple planes. The resulting cell aggregates are irregular in shape and appear more unicellular than filamentous. Some cells undergo a series of divisions in one plane to produce short motile hormogonia..
.... Genus *Chlorogloeopsis*
A2. True branching results from lateral division along main axis of the filamcent .... *See* B.

B1. Cells of lateral branches are morphologically different from the cells in the main axis............. Genus *Fischerella*
B2. Cells of lateral branches are not morphologically different from the cells in the main axis..................... *See* C.

C1. Trichomes uniseriate throughout................................
.... Genus *Hapalosiphon*
C2. Trichomes multiseriate, at least in part......................
.... Genus *Stigonema*

The genus *Chlorogloeopsis* is a member of the Stigonematales even though it never displays a branched filamentous habit characteristic of the other genera in this order (Fig. 7). It does however produce typical, short-chained, motile hormogonia that differentiate into multiseriate-cell aggregates that appear more unicellular than filamentous.

The genera *Fischerella* and *Hapalosiphon* are both capable of true branching (Fig. 7). In *Fischerella* the cells of the lateral branches differ in size from the cells in the main axis and are uniseriate, whereas the main axis may become multiseriate. In *Hapalosiphon* both the main axis and lateral branches are uniseriate and composed of similar sized cells.

Members of the genus *Stigonema* are also capable of true branching. *Stigonema* is distinguished from *Fischerella* and *Hapalosiphon* by the complexity of its thallus structure resulting from an ability to form both multiseriate and uniseriate branches. Only one strain of *Stigonema minutum* has been cultured (Zehnder, 1985). There are also a number of genera in this order that have not yet been cultured which will make fascinating subjects for future study (Geitler, 1932).

Representatives of the genera *Anabaena, Cylindrospermum, Calothrix,* and *Fischerella* are illustrated in The Phototraphic Prokaryotes in the second edition, Figs. 14–17.

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CHAPTER 2.2

The Cyanobacteria—Ecology, Physiology and Molecular Genetics

YEHUDA COHEN AND MICHAEL GUREVITZ

Introduction

The cyanobacteria and prochloroaceae are the only prokaryotic groups that share the use of photosystems I and II and hence the ability to carry out oxygenic photosynthesis with all photosynthetic eukaryotic organisms (Stanier, 1977). The structure of the reaction center complexes seems to be evolutionarily conserved in all these organisms, but there is a large diversity in their antenna chlorophyll complexes (Glazer, 1983). Cyanobacteria are unique since they are also capable of anaerobic metabolism, and several groups among the cyanobacteria share with the prokaryotic anoxygenic phototrophic bacteria the ability to perform anoxygenic photosynthesis using reduced electron donors (Cohen et al., 1975a; Padan and Cohen, 1982; Cohen et al., 1986; de Wit and van Gemerden, 1989; Garcia-Pichel and Castenholz, 1990). They also share with many archaebacteria the ability to use elemental sulfur for anaerobic dark respiration (Oren and Shilo, 1979).

Cyanobacteria are a diverse group of oxygenic photosynthetic prokaryotes, exhibiting versatile physiology and wide ecological tolerance that contribute to their competitive success over broad spectrum of environments both planktonic and benthic (Shilo, 1989). They are dominant in a broad spectrum of terrestrial habitats, including deserts, where they are found as endoliths in the antarctic dry valleys and cold deserts (reference is not an exact matchFriedman, 1982); hot deserts in the Sahel (Potts and Friedmann, 1981); tropical rain forests; and mangrove swamps. Planktonic cyanobacterial blooms are common in freshwater bodies, especially where eutrophic conditions exist (hyperscums of *Microcystis aeruginosa* over 1 m thick have been reported, Zohary and Cohen, 1991), as well as in oligotrophic oceans (where *Synechococcus* spp. may account for 50% of the chlorophyll *a* biomass, Waterbury et al., 1979; Olson et al., 1990). Among this widespread group of photosynthetic prokaryotes are species that are able to grow as aerobic photoautotrophs; anaerobic photoautotrophs using either H_2S, thiosulfate, or H_2 as alternative electron donors; photoheterotrophs; chemoheterotrophs; and as anaerobic or aerobic dinitrogen fixers (Allen, 1985; Paerl et al., 1989; Stal et al., 1989b).

Historically, cyanobacteria were long classified as blue-green algae according to the Botanical Code, and it was only in the eighth addition of *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons, 1974) that they were first assigned to a separate division of the prokaryotes.

Cyanobacteria are unique in that they are Gram-negative prokaryotes and yet perform oxygenic photosynthesis very similar to that of higher plants. Thus, they may serve as a model system to resolve biological questions difficult to approach in higher plants, and they can be target organisms for research not directly related to photosynthesis. Hence, not only questions associated with the photosynthetic apparatus and function, carbon fixation, light-regulated gene expression, but also cell differentiation and resistance to environmental factors or stress may be easier to address with the power of molecular genetics in cyanobacteria, rather than in higher plants.

The key to the genetic study and manipulation of cyanobacteria is the development of a transformation system (either natural or artificial) for DNA uptake. Such a system of cellular competence could be used to internalize exogenous DNA with or without a special treatment to the recipient cells prior to their incubation with the donor DNA. The majority of cyanobacterial strains capable of DNA-mediated transformation possess physiological, or natural, competence for DNA uptake. The mechanism involved in such a process is still unknown but the phenomenon has been extensively used to transform cyanobacterial cells either chromosomally or with plasmids.

Chromosomal transformation is achieved by recombination and internalization of donor chromosomal DNA with homologous DNA in

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the recipient cell. By using donor DNA that has been subjected prior to the transformation to either in vivo mutagenesis or to recombinant DNA manipulations in vitro, it is possible to create and easily select for mutants according to their phenotype or a genetic marker. It is thus possible to produce insertion or deletion mutations in cyanobacterial genomes, permitting the analysis of gene structure, function, and organization.

Plasmid transformation involves the introduction of a plasmid DNA molecule able to replicate in the recipient cell. This allows development of plasmid vectors for cyanobacteria that can be used to produce cyanobacterial partial diploids or meroploids, as well as to introduce foreign genes whose function is analyzed in the recipient cell.

Cyanobacteria can also be transformed with nonreplicating plasmids containing chromosomal-homologous sequences. In such a case, except for homologous recombination between the plasmid and the chromosome, another outcome may be the incorporation of the entire plasmid into the cyanobacterial chromosome if a single recombination event occurs between the homologous sequences. By constructing such mutants, it is possible to create heterogenotes in which a foreign mutant gene is tandemly placed next to the wild-type gene in the chromosome.

So far, only unicellular strains have been found to possess a natural competence for DNA uptake. However, the development of conjugal DNA-mediated transfer for filamentous cyanobacteria has circumvented this problem and opened a way to introduce genetic material into cells that were previously restricted for genetic transformation. The shuttle vectors developed for such transformation are capable of replicating in both *Escherichia coli*, in which DNA manipulations can be performed, and in the recipient cyanobacterium. Another way to introduce DNA into cyanobacteria could exploit cyanophages as candidate vectors. Despite the fact that several cyanophages have been described, no gene transduction has been reported in cyanobacteria.

Our present understanding of the ecology, physiology, and molecular genetics of cyanobacteria allows the exploration of the use of cyanobacteria for several biotechnological uses, such as the production of specific photosynthetic pigments and herbicides and the use of cyanobacteria for agricultural dinitrogen fixation.

Cyanobacterial Mats

Cyanobacterial mats are stratified benthic microbial communities in which cyanobacteria are dominant, developing at sediment-water interfaces in a wide spectrum of shallow aquatic systems. They form laminated multilayers (biofilms) embedded in copious amounts of polysaccharides excreted by the benthic microbial community (Krumbein et al., 1977). The laminated microbial community develops in response to chemical microgradients at the mat surface. Such gradients can be measured by the use of specific microelectrodes for pO_2, pH, and pS^{2-} (Jørgensen et al., 1979b, 1983; Revsbech et al., 1983; Revsbech and Jørgensen, 1986). Steep microgradients of oxygen from zero to 1000 μM, pH from 7.0 to over 11.0, and H_2S from zero to 500 μM can be found in the upper 2 mm of the cyanobacterial mat. These chemical microgradients are largely the result of intensive oxygenic photosynthesis, which takes place at the very surface of the cyanobacterial mat. Light is rapidly absorbed by the benthic cyanobacterial community, leading to a steep gradient of photosynthetically available light (Jørgensen et al., 1987; Jørgensen, 1989). Sulfide production and oxidation is tightly coupled to the excretion of organic photosynthates by the cyanobacteria, resulting in maximal rates of sulfate reduction within the photic zone at midday (Cohen, 1984b). The concomitant activities of oxygenic photosynthesis, together with anaerobic metabolism of anoxygenic photosynthesis by associated anoxygenic phototrophic bacteria, such as *Chloroflexus* spp., *Ectothiorhodosira*-like spp., and *Thiocapsa* spp. (Stolz, 1984; D'Amelio et al., 1987), as well as sulfate reduction (Jørgensen and Cohen, 1977), and methane production (Cohen, 1986), may be possible by H_2 production through photoreduction of water by the cyanobacteria at high light intensity and under CO_2 limitation. It is probably CO_2 limitation at the photosynthetically active zone of the cyanobacterial mat that causes isotope fractionation for $^{13}C/^{12}C$ in the organic matter in shallow-water hypersaline microbial mats to be relatively heavy (–4% to –11%) (Des Marais et al., 1989). Addition of CO_2 results in a drastic enhancement of the rate of CO_2 photoassimilation by the cyanobacterial mat community (Rothschild and Mancinelli, 1990).

Because the chemical microgradients are largely light-dependent (Cohen, 1984b), the cyanobacterial mat becomes completely anoxic at night and thus exposed to elevated H_2S concentration at about neutral pH. Cyanobacteria may cope with these diurnal fluctuations of oxygen and sulfide by either migrating vertically in the mat and thus avoiding the exposure to toxic levels of sulfide at lower light intensity (Castenholz, 1973; Richardson and Castenholz, 1989) or by shifting their photosynthetic mode to adapt to life under sulfide (Cohen, 1984a).

Highly developed cyanobacterial mats are common in hot springs at temperatures of less than 74°C and at pH levels above 5.0 (Castenholz, 1984; Ward et al., 1989), in alkaline lakes, and in marine (e.g., Bauld, 1984) and hypersaline ecosystems at salinities of up to 20% salt (Des Marais et al., 1989). Cyanobacterial mats at salinities lower than 4.5% rarely accumulate because they are heavily grazed by a variety of higher organisms. Only under extreme environmental conditions of elevated temperatures and/or high salinity do cyanobacterial mats accumulate (to over 1 m thick in in environments such as the hypersaline Solar Lake on the coast of the Gulf of Aqaba in Sinai; Krumbein et al., 1977).

Cyanobacterial mats are probably the oldest form of known life on earth, as shown by the oldest known microfossils which were found in lithified microbial mats (stromatolites) in Central Australia and have been dated at 3.56 billion years (Knoll, 1989). Stromatolites are the most dominant sedimentary structures in rocks throughout the Precambrian era, together with vast deposits of Banded Iron Formation, both of which are associated with widespread development of cyanobacterial mats. While most researchers agree that these sedimentary rocks are associated with the development of microbial mats, many dispute the interpretation of cyanobacterial mat communities, mainly because 16S rRNA analyses of cyanobacterial cultures suggest these organisms are more recently evolved (Woese, 1987; Turner et al., 1989).

Marine and hypersaline cyanobacterial communities are often covered with thin layers of eukaryotic diatoms, *Navicula*, *Nitzchia*, and *Amphora* spp. The cyanobacterial mats in marine and hypersaline environments consist primarily of the filamentous cyanobacteria *Microcoleus chthonoplastes*, *Oscillatoria limnetica*, *Phormidium* spp., *Oscillatoria* spp., *Lyngbia* spp., *Calotrix* spp., *Spirulina* spp., and *Schytonema* spp. They are often associated with a variety of unicellular cyanobacteria, of which the dominant types are *Synechococcus* spp., *Synechocystis* spp., *Pleurocapsa* spp., and *Dermocarpa* spp. (Jøorgensen et al., 1983; Stolz, 1984; D'Amelio et al., 1989). The unicellular forms are more abundant at salinities higher than 10%. These cyanobacterial communities occupy the photic zone, which may range from less than 2 mm to over 6 cm. These cyanobacteria are closely associated with a variety of anoxygenic phototrophs, many of which are not yet available in pure culture, including several morphotypes of the green gliding phototrophic bacteria (*Chloroflexus* spp.) and filamentous purple bacteria resembling *Ectothiorhodospira* (D'Amelio et al., 1987, 1989). Other anoxygenic phototrophic bacteria found in hypersaline mats include *Chromatium* spp., *Thiocapsa pfennigii*

(Stolz, 1984), and *Ectothiorhodospira halochloris* (Imhoff et al., 1978). The assemblage of the various anoxygenic phototrophic bacteria are found within the cyanobacterial layer or right below at the deeper part of the photic zone, where a distinct olive-green and/or purple layer can often be found. Right below this layer, a distinct microzone of chemotrophic *Beggiatoa* spp. and *Thioploca* spp. is characteristic for the chemocline, where both oxygen and sulfide coexist (D'Amelio et al., 1989).

In hot spring cyanobacterial mats, where chemical composition and temperature vary widely (Brock, 1978), cyanobacteria are excluded at temperatures above 74°C and at acidic pH (Brock, 1973). *Synechococcus lividus* forms a thick layer over a distinct accretion of *Chloroflexus auranticus* at temperatures of 74 to 60°C. Only at lower temperature can a deep brownish red, c-phycoerythrin-containing cyanobacterium, *Oscillatoria terebriformis*, be found. At temperatures below 45°C, the moderately thermophilic cyanobacteria *Phormidium, Pseudoanabaena, Synechococcus, Calotrix*, and *Mastigocladus laminosus* are found (Castenholz, 1984).

Nitrogen fixation was demonstrated in the mat-forming cyanobacteria *Oscillatoria limosa* (Stal and Krumbein, 1985; Stal et al., 1989) and *Microcoleus chthonoplastes* (Pearson et al., 1981).

Cyanobacterial mats developing in extreme conditions of elevated temperature or at high salinity are clearly laminated microbial communities that can serve as good models for the study of metabolic and genetic interactions in a multi-layered microbial biofilm. The various aspects of the ecology, physiology, and molecular biology of both natural communities of cyanobacterial mats as well as isolated pure cultures from microbial mats are discussed in Cohen et al. (1984b) and Cohen and Rosenberg (1989).

Anoxygenic Photosynthesis in Cyanobacteria

Cyanobacteria are often found to develop in environments rich in sulfide. These environment are also rich in available nitrogen and phosphate and CO_2 sources as the result of the anaerobic breakdown of organic matter (Cohen, 1984b). Sulfidic environments may also provide a low-oxygen environment suitable for an optimal operation of ribulose bisphosphate carboxylase (rubisco), the key enzyme of CO_2 photoassimilation (Avron, 1989). Yet H_2S is highly toxic and brief exposure to low sulfide concentrations may irreversibly inhibit most eukaryotic phototrophs. Even sulfide-producing prokaryotes, such as sul-

fate-reducing bacteria, are inhibited at elevated H₂S concentrations.

Nakamura (1938) was the first to observe growth of *Oscillatoria* in 1 mM H_2S without the detection of oxygen production and the deposition of sulfur in the cells, suggesting that H_2S was being used as an electron donor for CO_2 photoassimilation. Cohen et al. (1975a, 1975b) confirmed this observation in cultures of *Oscillatoria limnetica* isolated from the hypolimnion of a stratified hypersaline lake in Sinai, where this cyanobacterium was the dominant phototroph at up to 3 mM H_2S (Cohen et al., 1977). This cyanobacterium was found to carry out anoxygenic CO_2 photoassimilation with sulfide as an alternative electron donor to water in a photosystem I (PS-I)-driven reaction similar to that carried out by anoxygenic photosynthetic bacteria. Assimilation of CO_2 in the presence of sulfide was found to involve a stoichiometric oxidation of H_2S to elemental sulfur, which was deposited outside the cells (Cohen et al., 1975). This process was independent of photosystem II (PS-II), which was found to be fully inhibited at low sulfide concentrations of 0.1–0.2 mM. Anoxygenic photosynthesis in this organism required an induction period of 2 h in the light and in the presence of sulfide. Photosynthetic efficiency of H_2S-driven anoxygenic photosynthesis was found to be even higher than oxygenic photosynthesis, especially when exposed to red light, and could sustain growth at a doubling rate of about 6 h (Oren et al., 1977; Oren and Padan, 1978). A specific sulfide-quinone reductase was needed for the induction of anoxygenic photosynthesis (Arieli et al., 1991). These workers demonstrated that photosynthetic thylakoids isolated from *Oscillatoria limnetica* grown under sulfide have the capacity in the dark to catalyze electron transfer from sulfide to externally added quinones. They therefore proposed that a membrane-bound sulfide-quinone reductase is the sulfide-induced factor which enabled the use of sulfide for anoxygenic photosynthesis in *Oscillatoria limnetica*. The detailed molecular mechanism of electron transport coupled to proton translocation in cytochrome b_6f/bc_1 complexes is not yet understood, but cytochrome b_6f may be involved in anoxygenic photosynthesis. Arieli et al. 1991 hypothesize the existence of several electron carriers which mediate electron transfer from different donors to the plastoquinone-b_6f complexes. These carriers are thought to have a common site, which interacts with the plastoquinone or cytochrome b_6, as well as a different site, which interacts with the various different electron donors.

Sulfide was found to be oxidized to thiosulfate in *Microcoleus chthonoplastes* (de Wit and van Gemerden, 1987, 1989). Thiosulfate was found to be an electron donor (Castenholz and Utkilen, 1984). Sulfide could be replaced with molecular hydrogen as another alternative electron donor for H_2-dependent photoreduction of CO_2 in another PS-I-dependent anoxygenic photosynthesis in *Oscillatoria limnetica* and *Aphanotheca halophytica* (Belkin et al., 1988).

Depending on the reaction conditions, the electrons from sulfide in *O. limnetica* may be channelled to CO_2 photoassimilation, dinitrogen fixation, or hydrogen evolution under CO_2 limitation (Belkin, Shahak, and Padan, 1988). Sulfide stimulation of hydrogen production was also found in *Nostoc muscorum* by Weisshaar and Boeger (1983).

Cyanobacteria may evolve different mechanisms to grow in the presence of sulfide. *Oscillatoria* spp. grown in sulfide-rich hot springs in Utah were found to be able to carry out oxygenic photosynthesis even in the presence of 2 mM sulfide (Cohen et al., 1986), and similar behavior was found in cyanobacteria grown in sulfide-rich hot springs in New Zealand. In these organisms, PS-II is considerably less sensitive to sulfide toxicity compared to *Oscillatoria limnetica,* but the mechanism of sulfide detoxification is not as yet known. These organisms do not have the capacity for anoxygenic photosynthesis.

Microcoleus chthonoplastes, a cosmopolitan mat-building cyanobacterium found in marine and hypersaline environments, has been demonstrated to have both the capacity to carry out oxygenic photosynthesis at 1 mM sulfide concentration and to be able to carry out anoxygenic photosynthesis using sulfide as an electron donor (Cohen et al., 1986; Jorgensen et al., 1986; de Wit and van Gemerden, 1989).

Other Anaerobic Metabolism Pathways in Cyanobacteria

Nitrogen Fixation

Anaerobic conditions enhance dinitrogen fixation in cyanobacteria because of the toxicity of oxygen to the nitrogenase enzyme. Some cyanobacteria have developed specialized cells called heterocysts where PS-II is absent, but many cyanobacteria can fix dinitrogen aerobically without the need for heterocysts. Two main mechanisms for keeping oxygen away from the nitrogenase sites have been described: 1) Formation of oxygen-poor microniches in aggregates of cyanobacteria, where PS-II and oxygen evolution take place in the periphery of the aggregate while dinitrogen fixation occurs in the center of the cyanobacterial clump. Such a mechanism was described for aggregates of *Trichodesmium* (taxonomically, *Oscillatoria*), a widespread marine

filamentous cyanobacterium common in nitrogen-poor tropical oceans (Paerl et al., 1989b; Currin et al., 1990). Using immunoassays, Paerl and his colleagues have shown the localization of nitrogenase in these organisms in the center of the *Trichodesmium* aggregate (Paerl et al., 1989a). 2) Temporal separation of oxygen evolution and nitrogenase activity was demonstrated in several unicellular cyanobacteria, including the widespread picoplanktonic *Synechococcus* in tropical oceans (Mitsui et al., 1986) and the mat-forming cyanobacterium *Oscillatoria limosa* (Stal et al., 1989).

Anaerobic Dark Respiration and Fermentation

Several mat-forming cyanobacteria have been found to survive better under anaerobic conditions in the dark. This phenomenon was described for mats of *Microcoleus chthonoplastes* (Jørgensen et al., 1988), *Oscillatoria terebriformis* (Richardson and Castenholz, 1987), and *O. limosa* (Stal et al., 1989). Glycogen was found to be fermented in a heterofermentative mode producing equal amounts of lactate, ethanol, and CO_2 in *O. limosa* (Stal et al., 1989), while only lactate was found in anaerobic fermentation in *O. limnetica* (Oren and Shilo, 1979). Acetate production in anerobically grown *O. limosa* in the dark was postulated to be the result of anerobic degradation of trehalose (Stal et al., 1989), which serves as an osmolite in several cyanobacteria (Hershkovitz et al., 1991).

Addition of elemental sulfur to anaerobically grown *O. limnetica* and *O. limosa* resulted in anaerobic respiration and reduction of the elemental sulfur to sulfide in the dark (Oren and Shilo, 1979; Stal et al., 1989). The reduction of elemental sulfur in the dark to sulfide was demonstrated in the chemocline of Solar Lake, Sinai (Jørgensen et al., 1979), where a bloom of the unicellular cyanobacterium *Dactylococcopsis salina* (Walsby et al., 1983; van Rijn and Cohen, 1983) and *Aphanotheca halophytica* were dominant.

Given the widespread distribution of cyanobacteria in a spectrum of sulfide-rich environments, such as hot springs and cyanobacterial mats in marine and hypersaline environments, a variety of other low-redox, sulfur-dependent metabolic pathways are expected to be discovered in the future.

Cyanobacterial DNA

Like other prokaryotes, cyanobacteria contain two types of DNA, chromosomal DNA and smaller, autonomous, extrachromosomal molecules. Both types of DNA can be used for gene transfer and manipulation, each having both advantages and disadvantages. In both instances, transformability of the cell is a prerequisite for gene manipulations. The introduction of foreign DNA can be monitored either by following a genetic marker or by observing some phenotypic alteration indicating a successful transformation. Targeting of the foreign DNA into specific sites in the acceptor DNA and the copy number of the introduced gene may both be of great importance for its expression. Mobilizing DNA sequences into or out of a cyanobacterial cell is not always easier when using extrachromosomal self-replicating molecules. So far, no specific function has been assigned to cyanobacterial plasmids, and in some instances, curing the cells of their plasmids did not have any deleterious effect on the cells, nor was their viability harmed. Nevertheless, various procedures for transforming cyanobacteria have been developed using both types of cyanobacterial DNA.

Genome Size

The genome size of 128 strains representing all major taxonomic groups of cyanobacteria has been measured from the kinetics of renaturation of DNA. The range of size is between 1.6×10^9 Da in the unicellular strains, which is comparable in size to those of other bacterial genomes (Gillis et al., 1970; Wallace and Morovitz, 1973), and 8.6×10^9 Da, in the larger filamentous strains, which greatly exceeds the largest genome previously described in prokaryotes. Even though a larger genome might be anticipated in organisms capable of fixing nitrogen and exhibiting morphological differentiation, the great excess of DNA in these strains raises the possibility that some DNA sequences do not possess a coding capacity (Doolittle, 1979). Genetic mapping of the chromosome of cyanobacteria may shed light on such enigmas as well as establish a physical map of the chromosome. Wolk and coworkers have initiated such a study with *Anabaena variabilis* by producing a set of serially overlapping cosmid clones (Herrero and Wolk, 1986).

The genome sizes of cyanobacteria are discontinuously distributed into four distinct groups with means of 2.2×10^9, 3.6×10^9, 5.0×10^9, and 7.4×10^9 Da. This suggests that genome evolution in cyanobacteria occurred by a series of duplications of a small ancestral genome (Herdman et al., 1979).

Cyanobacteria contain several identical chromosomes in each cell (Williams, 1988), which suggests the possibility of interactions between them. Even though genome interaction seems very likely, no direct molecular evidence for

recombination between chromosomes in cyanobacteria has been documented. An intrachromosomal gene conversion mechanism involving the *psb*A gene family in *Synechococcus* PCC 7942 was elucidated by Brusslan and Haselkorn (personal communication). Interchromosomal recombination was observed in *Synechocystis* PCC 6803 by Gurevitz and co-workers (unpublished observations). Thus, the general assumption favors the existence of such mechanisms in cyanobacteria. The *rec*A gene was identified in some cyanobacterial species (Murphy et al., 1987), and in many instances, recombination between chromosomal DNA and plasmid DNA has been proposed. To minimize fortuitous results, this phenomenon should be taken into account whenever introduction of foreign or engineered genes into cyanobacteria is considered.

Plasmids

The existence of nonchromosomal DNA molecules in cyanobacteria was first observed in *Anacystis nidulans* (*Synechococcus* sp.) (Asato and Ginoza, 1973). Ever since, it has been evident that nearly all cyanobacteria possess endogenous plasmids (Rebiere et al., 1986), suggesting that it might be feasible to use these molecules as tools in molecular genetic studies. However, no function encoded by cyanobacterial plasmids has been identified so far, and the regulatory mechanism of their replication is still unknown. Several workers have tried to attribute various functions to plasmids, such as gas vacuolation (Walsby, 1977), toxin production (Hauman, 1981; Schwabe et al., 1988), resistance to high salt concentrations or heavy metals (Van den Hondel et al., 1979), resistance to antibiotics (Flores and Wolk, 1985), and synthesis of restriction/modification enzymes (Whitehead and Brown, 1985). It was observed that spontaneous loss of plasmids from different unicellular strains, such as *Synechococcus* sp. PCC 6301 and 73109 (Whitehead and Brown, 1985) and *Synechocystis* sp. PCC 6803 (Tandeau de Marsac and Houmard, 1987), could occur without causing any obvious phenotypic change.

Still, several research groups have produced various cloning vectors or shuttle vectors (*E. coli*-cyanobacterium) by utilizing these self-replicating molecules. Nevertheless, this approach is limited since the parameters related to mechanisms involved in plasmid replication and control of plasmid copy number in cyanobacteria remain obscure.

The number of autonomous plasmids per cyanobacterial cell may vary from one up to eight, with sizes ranging from 1.3 kb to about 130 kb (Tandeau de Marsac and Houmard, 1987).

In a few instances, different strains of cyanobacteria possess plasmids of identical size and endonucleolytic digestion pattern (Walsby, 1977; Hauman, 1981; Van den Hondel et al., 1979) or sequence homologies (Schwabe et al., 1988). Since some of these strains are of different geographical origins, an implication can be made that interspecific or even intergeneric plasmid transfer may occur in nature.

In other cases, multimeric forms of a single plasmid species were elucidated by digestion with restriction enzymes. Reaston et al. 1980 found in *Nostoc* sp. PCC 7524, three plasmids, pDU1, pDU2, and pDU3 (6.1, 11.8, and 37.3 kb respectively), where pDU2 is a dimeric form of pDU1. This raises questions in regard to the function and preservation of such forms in the cell, particularly if an active recombination mechanism followed by segregation is considered. Even though the plasmids obtained from different cyanobacterial species are generally similar when purified by different researchers, there are exceptions that in few instances were attributed in part to the extraction protocol used in the various laboratories. However, the very extensively studied cyanobacterium *Calothrix* sp. PCC 7601 (*Fremyella diplosiphon*) is a real exception since different restriction patterns and different plasmid numbers were obtained in various laboratories (Simon, 1978; Bogorad et al., 1983; Tandeau de Marsac and Houmard, 1987). These variations could not be explained or related to phenotypic changes of the cells. However, these results, together with previous observations (Tandeau de Marsac, 1983), that spontaneous pigment mutants arise with high frequency (10^{-3} to 10^{-4}) in this organism led the de Marsac group (Tandeau de Marsac and Houmard, 1987) to suggest the existence of mobile genetic elements in the *Calothrix* strain. So far, whether these elements might be transposons, insertion elements, or Mu-type phages has not been determined. If mobile DNA elements do exist, one would expect high frequencies of genome rearrangements in cyanobacteria. Nevertheless, the only rearrangement documented so far has been the well-analyzed rearrangement of the *nif* region in the filamentous dinitrogen-fixing strain *Anabaena* sp. PCC 7120, which accompanies the differentiation of vegetative cells into heterocysts (Golden et al., 1985a, 1985b).

Restriction/Modification of Cyanobacterial DNA

Chromosomal DNA from various unicellular and filamentous cyanobacteria is considered highly resistant to cleavage by a number of restriction endonucleases, although only a mod-

est repertoire of restriction enzymes was found in these organisms (Van den Honel et al., 1983; Herrero et al., 1984; Lambert and Carr, 1984). Still, more than 50 strains from nine different genera contain from one to five restriction endonucleases (Tandeau de Marsac and Houmard, 1987). Some of the endonucleases, such as the AvaI or AvaII type, are isoschizomers that are widely distributed among different genera. Presumably, this may be related to a common evolutionary origin. Together with their modification methylase associates, they could be acquired by the transfer of plasmid- or cyanophage-encoded genes. However, no correlation among the plasmid content of cyanobacterial strains, their phage sensitivity, and their restriction endonuclease content has been observed.

A similar pattern of DNA modification was found by Lambert and Carr (1984) in diverse filamentous strains that contain (*Gleotrichia* and *Plectonema*) or do not contain (*Nostoc*) different types of restriction endonucleases. Concomitantly, they observed a great variation in the susceptibility of genomes from five unicellular strains, to cleavage by a group of restriction enzymes. By using isoschizomers, Geier and Modrich (1979) were able to demonstrate that DNA resistance to cleavage could originate either from a host-controlled restriction/modification system, as found in other groups of bacteria, or due to a *dam* (DNA adenine methylase) enzyme. Examples of possible restriction/modification systems operating in vivo were depicted for *Anabaena* sp. PCC 7937 (Currier and Wolk, 1979) and for *Synechococcus* sp. PCC 6301 (Szekers, 1981; Szekers et al., 1983). Cyanophage N-1 derived from *Anabaena* sp. PCC 7120 was able to form plaques on *Anabaena* sp. PCC 7937 at a very low efficiency, but the surviving progeny of the phage increased its plaque formation efficiency when reinfecting the same strain (*Anabaena* sp. PCC 7937). The conclusion from this experiment was that the cyanophage N-1 DNA that escaped restriction in *Anabaena* sp. PCC 7937 could have been modified by the host cell. On the other hand, in experiments performed in vitro by Szekers (1981), it was shown that DNA from *Synechococcus* sp. PCC 6301 was cleaved by an endonuclease produced by AS-1 cyanophage-infected cells. Moreover, the cyanophage DNA was unprotected after cloning in a plasmid vector propagated in an *E. coli* strain. These results indicated that a restriction/modification system was induced in *Synechococcus* sp. PCC 6301 by AS-1 cyanophage; the modification mechanism involved remains unknown.

Another example is the modification enzyme M.NspMACI, which was purified from *Nostoc* sp. PCC 8009. This strain normally contains an isoschizomer of BglII (Reaston and Carr, 1985) called NspMACI, which is unable to cleave DNA previously modified by M.NspMACI while BglII does. This is an indication that the modification sites affected by M.BglII and M.NspMACI are different.

Several explanations for the resistance to cleavage of cyanobacterial DNA were proposed (Lambert and Carr, 1984; Jager and Potts, 1988), including the presence of specific inhibitors like DNA-binding proteins, unusual bases (other than MeC and MeA), and the absence of specific recognition sequences in the genomic DNA of certain cyanobacteria. Whether such modification processes play a role in cyanobacterial gene expression and cell differentiation requires further examination.

Genetic Recombination

Cyanobacteria are susceptible to ultraviolet (UV) irradiation, and therefore they are expected to possess an efficient mechanism to permit repair of UV-induced damage (by genetic exchange and gap-filling). The occurrence of homologous recombination in cyanobacteria explains the high efficiency of foreign DNA incorporation into the genome of unicellular strains like *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803. Recombination activity in cyanobacteria is advantageous for genetic engineering when utilized for the internalization of exogenous DNA into the chromosome. It may be considered a disadvantage when such activity may destroy engineered constructs mobilized into cyanobacteria via plasmid vectors. On one hand, homologous recombination has become a useful way to achieve insertions or deletions at various genes. However, one of the major problems associated with the introduction of cloning vehicles into cyanobacteria is recombination between vector and resident plasmids. For the stable maintenance of hybrid plasmids in *Synechocystis* PCC 6803, some Rec⁻ mutants deficient in homologous recombination were employed. In these mutants, no transformation with either chromosomal markers or integrative vectors occurred, while the autonomously replicative vector pSE76 transformed such recipients at high frequency (Shestakov et al., 1985). Although all transformable cyanobacteria are recombination-proficient, no documentation in respect to the molecular mechanism involved has been reported. However, the *rec*A gene from *Synechococcus* PCC 7002 was identified, and its sequence determined (Murphy et al., 1987). This problem is significant and should be addressed when planning experiments of complementation, of DNA transfer (when recombination activity should be avoided), and of studies related to

dominance of genes (Haselkorn, 1985). Cyanobacteria possess several identical chromosomes per cell. When transformed with exogenous DNA, in most cases, all the chromosomes will contain the foreign DNA after a few cell cycles, due to segregation. Whether this is followed by interchromosomal gene conversion is still unknown. A concomitant unsolved question is related to the possible occurrence of intrachromosomal gene conversion in cyanobacteria. In addition, it is still unknown whether there exists a mechanism for gene duplication or amplification in cyanobacteria. It is suspected that such a mechanism does exist, particularly as a response to environmental stimuli like an excess of light. Such processes rely on recombination activity, and their better understanding is obviously necessary.

Gene Transfer in Cyanobacteria

To mobilize genetic material into cyanobacteria, the target cyanobacterium should: 1) be transformable by exogenous DNA; 2) have no host restriction; 3) be Rec⁻ when stabilization of autonomous vector is required; 4) allow the generation of meroploids; 5) express genetic markers; and 6) recognize regulatory elements required for transcriptional and translational activities.

A few unicellular strains were found to be competent for genetic transformation and can be readily transformed with either circular or linear DNA. The donor DNA may contain a cyanobacterial origin of replication (*ori*), thus becoming a replicon within the transformed cells, or be lacking an *ori*. In either case, the fate of this DNA inside the transformed cell (recipient) may be different. A more sophisticated approach was required for the development of gene transfer mechanisms in filamentous strains.

A recombinant plasmid, pCH1 (Ap^R), derived from the endogenous pUH24 plasmid of *Synechococcus* PCC 7942 and possessing the Tn*901* transposon, was the first used by Van den Hondel and co-workers (1980) to transform *Synechocystis* PCC 7942. Transformed cyanobacteria grew in presence of ampicillin but the vectors used were unable to replicate in *E. coli*, which limited their construction and utilization. Since recombinant DNA technology has been largely developed using *E. coli*, it became a great advantage to have shuttle vectors capable of replicating both in *E. coli* and in cyanobacteria. A few cyanobacterial plasmids were used to construct a variety of shuttle vectors (Pouwels et al., 1985; Tandeau de Marsac and Houmard, 1987).

The pCH1 plasmid was further used to create a deletion derivative (pUC1) lacking one of the

inverted repeats of Tn*901*. This construction stabilized the marker and prevented any further transpositions. From pUC1 (Ap^R), a family of shuttle vectors was prepared by Van Arkel and colleagues (Kuhlemeier et al., 1981) by combining it with the *E. coli* plasmid pACYC184 (Cm^R). The resulting shuttle vector pUC104 (Ap^R, Cm^R), was stably maintained in *Synechococcus* PCC 7942. Shuttle vector pUC104 purified from *E. coli* was less efficient in transforming the cyanobacterium as compared to pUC104 purified from the cyanobacterium itself. It was hypothesized that there was a difference in their post-replicational DNA modifications (Kuhlmeier et al., 1981). This idea was supported by Gallagher and Burke (1985), who found a sequence-specific endonuclease, AniI, that could restrict foreign DNA. Plasmid pUC104 and lambda DNA were combined to construct a cosmid vector (pPUC29) capable of being packaged in vitro into lambda phage particles and of being introduced into *E. coli* by infection. A further step to optimize the host-vector system for gene cloning in *Synechococcus* PCC 7942 was carried out by Kuhlemeier et al. 1983, who isolated a host strain (R2-SPc) cured of the endogenous plasmid pUH24. A new construct, pUC303, has allowed cloning of several genes, including genes involved with nitrate reductase activity.

A limitation to gene cloning in autonomously replicating vectors may arise from recombination between two homologous genes located in the plasmid and in the chromosome. This limitation can be avoided by dual selection for both the vector and for the chromosomal insert (Kuhlemeier et al., 1985).

Other shuttle vectors for *Synechococcus* PCC 7942 were constructed with pUH24 plasmid and *E. coli* plasmid of the pBR family. The hybrid shuttle vector pLS103 (Cm^R) was constructed from pUH24 and pBR322 by Sherman and Van de Putte (1982). The plasmid pECAN1 (Ap^R, Cm^R), based on pBR325, was constructed by Gendel et al. (1983).

Most of these shuttle vectors contain just a few unique cloning sites. Therefore, a series of hybrid plasmids with multiple cloning sites were produced by Gendel et al. (1983). These vectors, the pPLANB series, provided five or seven unique sites and were able to transform both *E. coli* and *Synechococcus* PCC 7942 with high frequency (10⁻⁵ transformants/cell). Similarly, Lau and Straus (1985) constructed several small, versatile, shuttle vectors: Plasmid pXB7 (Ap^R) with 10 unique cloning sites; pECAN8 (Ap^R), with four sites within the *lac*Z gene, which permits easy detection of recombinants in the presence of X-gal in some *E. coli* strains and pKBX (Km^R) with 10 cloning sites.

Since the large plasmid of *Synechococcus* PCC 7942, pUH25, contains an origin of replication compatible with that of pUH24, Laudenbach et al. (1985) constructed the pANLO1a and pANLO1b shuttle vectors. Cotransformation with both types of replicons allows simultaneous cloning of foreign genes into the cyanobacterium.

Although great attention was directed towards the cyanobacterium *Synechococcus* PCC 7942, hybrid plasmids were constructed with the small, cryptic plasmid pAQ1 from *Agmenellum quadruplicatum* PR-6 as well. Buzby et al. (1983) produced the pAQE2 and pAQE10 from pAQ1 and pBR322 and pBR325 respectively. The PR-6 strain contains the restriction enzyme AquI (AvaI) and, as demonstrated with the R-2 strain, shuttle vectors purified from PR-6 had higher transformation efficiencies than vectors propagated in *E. coli*. It is likely that the cyanobacterial DNA was modified to resist AquI. A significant difference between PR-6 and R-2 strains is the fact that dimeric and trimeric forms of biphasic plasmids yielded higher transformation frequencies than the analogous monomeric forms (Buzby et al., 1983). By contrast, in R2 strain, monomers are more efficient for transformation. The shuttle vector pAQE17 constructed by Buzby et al. (1985) was used to express allophycocyanin genes from the cyanelle *Cyanophora paradoxa* in *Synechococcus* PCC 7002.

The similarity of the photosynthetic apparatus between cyanobacteria and higher plants is of great advantage for a molecular genetic approach to various constituents involved in this system. Thus, an heterotrophic organism could be beneficial when photosynthetic gene manipulation is required without deleterious effects on the investigated organism. Since the cyanobacterium *Synechocystis* PCC 6803 is a facultative photoheterotroph and amenable for genetic transformation, measures to develop a host-vector system with this organism were taken. The small endogenous plasmid, pSS2, derived from this strain was used to create shuttle cloning vectors (Shestakov et al., 1985; Chauvat et al., 1986). The plasmid pSE176 (Cm^R, Km^R), was constructed from pSS2, pACYC184, pUC4K and contained several restriction sites at the Km^R region plus the EcoRI site in the Cm^R gene. Chauvat and co-workers (1986) have also used pSS2 but with pACYC177 and constructed the hybrid plasmid pFCLV7 (Cm^R, Km^R).

Attempts to transfer plasmids of *E. coli* into cyanobacteria were unsuccessful, probably due to the fact that the origin of replication of these plasmids was not recognized by cyanobacterial replication factors. However, McFadden and coworkers reported the incorporation of pBR322 plasmid into permeaplasts of *Synechococcus* PCC 6301 (Daniell et al., 1986). Permea-plasts are potentially viable cells with high permeability and capacity for cell-wall regeneration. Therefore, they may take up DNA at elevated ratios and subsequently repair and divide. In another experiment, this group demonstrated the uptake and expression of foreign DNA within whole cells of *Synechococcus* PCC 6301 (McFadden and Daniell, 1988). The mechanism involved in the incorporation and expression of the foreign DNA in these cyanobacteria has to be clarified.

A simple method to introduce foreign DNA into the chromosome of unicellular cyanobacteria was developed by Williams and Szalay (1983). They constructed chimeric DNA in an *E. coli* vector consisting of a DNA fragment derived from *Synechococcus* PCC 7942 that had been interrupted by the foreign (donor) DNA fragment aimed for introduction into the cyanobacterium. This construct could propagate in *E. coli* and transform cyanobacteria. The transformation was achieved via double recombination between homologous chromosomal and plasmid DNA sequences. This allows site-directed insertion of foreign DNA into the cyanobacterial chromosome. Addition of a selectable marker to the foreign DNA sequence enabled an easy selection of recombinant cells. Furthermore, in a case where a single recombination event took place, the entire vector could integrate into the chromosome. Distinction between both cases could be achieved by growing the transformants in the presence of one antibiotic (double recombination) or two antibiotics (single recombination). This method was adapted for *Synechocystis* PCC 6803 (Williams, 1988; Shestakov et al., 1985) and is currently widely utilized for studying a variety of cyanobacterial genes via their insertional inactivation, deletion, or modification, or the expression of foreign genes in cyanobacteria.

DNA from *E. coli* can be transferred into unicellular cyanobacteria in various ways that depend on the natural competence of the recipient strains. Some of the filamentous strains, such as *Anabaena* and *Nostoc,* possess unique systems related to differentiation and nitrogen fixation, which are very attractive for molecular studies. Unfortunately, the amenability of these systems to genetic manipulations is limited. For such strains, a gene-transfer system is of great advantage. Wolk and co-workers developed a very elegant conjugal system for transfer of exogenous DNA into *Anabaena* PCC 7120 (Elhai and Wolk, 1988). Three elements were required for the conjugation process: 1) a shuttle vector capable of replicating in *E. coli* cells and cyanobacteria which carries a genetic marker expressible in cyanobacteria and without too many AvaI and AvaII restriction sites; 2) a colicin K or colicin D

plasmid capable of mobilizing the shuttle vector in *trans*; and 3) an IncP plasmid, such as RP4, R702, R751, and R7K, capable of mediating the transfer of DNA into a wide range of Gram-negative bacteria including cyanobacteria. This process necessitates conjugal contact since the DNA is transferred via the pili of the conjugants. Practicably, a colicin-containing strain of *E. coli* is transformed with the shuttle plasmid, then mated with the RP4-containing *E. coli* strain. The mating mixture is put on top of the recipient cyanobacteria and transferred after one day to selective medium. Green recombinant colonies can be detected within 10 days.

The DNA transfer by conjugation method has been applied to other strains as well. Bullerjahn and Sherman (1985) constructed a plasmid containing the colE1 *ori*, RP4 *tra* functions, and transposon Tn*501* (conferring resistance to mercuric ions). In *E. coli*, low concentrations of Hg^{2+} promote transposition and the same phenomenon is seen in *Synechocystis* PCC 6714; there, a low level of Hg^{2+} present during conjugation resulted in a fivefold increase in the frequency of obtaining Hg^{2+}-resistant exconjugants. The integration of Tn*501* into the cyanobacterial chromosome was verified by Southern blotting. This demonstrated the feasibility of using the conjugal DNA-transfer method with unicellular cyanobacteria.

Isolation of Cyanobacterial Genes

To analyze gene structure and function, it is crucial to isolate the gene of interest. The following methods have been employed for the isolation of cyanobacterial genes in recent years.

Direct Cloning by Phenotypic Complementation

This method utilizes the phenotypic complementation of *Escherichia coli* or cyanobacterial mutants. The approach involves selection with mutants maintained under permissive growth conditions, e.g., either permissive temperature for temperature-sensitive mutations or photo-heterotrophic or heterotrophic conditions for strains capable of these metabolic modes. Shotgun-cloned genomic DNA fragments from the wild-type strain can be introduced into the mutant by using a suitable vector. After a certain period under nonselective conditions to allow expression of marker genes associated with the introduced DNA, the cells are shifted to nonpermissive, selective conditions. Complementary DNA fragments may then be identified due to their ability to allow growth of mutants under otherwise nonpermissive conditions. Comple-

mentation is then verified by isolation of the recombinant plasmid and its introduction into the mutant, which should result in the phenotypic difference previously observed.

This general strategy was employed in cloning DNA fragments conferring herbicide resistance in *Synechococcus* sp. PCC 7002 (Buzby et al., 1987) or DNA fragments that complemented photosynthetically impaired mutants of *Synechocystis* sp. PCC 6803 (Dzelzkalns and Bogorad, 1987). A distinct advantage of this method, that is not available in eukaryotic systems, is that it can be used to isolate DNA fragments complementing mutations in components very difficult to identify and isolate. Also, it can be used to identify genes whose products are not structural cell components and therefore cannot be detected in typical purified preparations of cellular fractions or complexes. In rare instances, genes encoding peripheral polypeptides to the photosynthetic process may be cloned by complementation of characterized mutations in *Escherichia coli*. As an example, this strategy was employed in cloning the phosphoenolpyruvate carboxylase (*pcc*) gene of *Synechococcus* sp. PCC 6301 (Kodaki et al., 1985), and the *gln*A gene of *Anabaena* sp. PCC 7120 (Fisher et al., 1981).

Heterologous Hybridization

This method for isolation of cyanobacterial genes uses heterologous hybridization with cloned DNA fragments from prokaryotes or eukaryotes. It is probably the most widely applied method simply because of the sequence conservation within many genes of various phylogenetic origins whose product is functionally similar, thus preserving common structural characteristics and DNA sequence.

In higher plants, many components of the photosynthetic apparatus are encoded by chloroplast genes. The complete nucleotide sequence of two chloroplast genomes was determined, and the open reading frames (ORFs) and unidentified reading frames (URFs) were extensively analyzed (Ohyama et al., 1986; Shinozaki et al., 1986). Also, many nuclear-encoded polypeptides which are part of the photosynthetic apparatus were identified, and their genes or cDNA clones isolated (Coruzzi et al., 1983; Smeekens et al., 1985a, 1985b; Tittgen et al., 1986). The availability of characterized genes or cDNA clones from higher plants that play a role in photosynthesis provides a ready source of materials for isolation and subsequent manipulation of their closely homologous cyanobacterial counterparts. A large number of genes encoding components of the photosynthetic apparatus in cyanobacteria were cloned using heterologous hybridizing

probes. In most cases, these probes were derived from cloned genes related to the chloroplast genome. For example: *psa*A and *psa*B (Cantrell and Bryant, 1987b; Lambert et al., 1985) related to the PS-I complex; *psb*A (Curtis and Haselkorn, 1984), *psb*B (Vermaas et al., 1987), *psb*C and *psb*D (Williams and Chisholm, 1987), *psb*E and *psb*F (Pakrasi et al., 1988) related to the PS-II complex; *pet*A, *pet*B, *pet*D (Kallas et al., 1987), and *pet*F (Van der Plas et al., 1986) related to the electron transfer chain between both photosystems; *atp*A, *atp*B, *atp*E, *atp*H, and *atp*I (Cozens and Walker, 1987; Curtis, 1987; Lambert et al., 1985) related to the ATP synthase complex in the photosynthetic membrane; and *rbc*L and *rbc*S (Curtis and Haselkorn, 1983; Shinozaki et al., 1983; Nierzwicky-Bauer et al., 1984; Shinozaki and Sugiura, 1983) for rubisco.

Low-stringency hybridization with heterologous probes was also successfully employed to isolate members of the phycobiliprotein or linker-polypeptide multigene families (Conley et al., 1985, 1986; Houmard et al., 1986; Mazel et al., 1986; Dubbs and Bryant, 1987; Belknap and Haselkorn, 1987; Lemaux and Grossman, 1985). With this type of hybridization, sometimes it is possible to obtain positive results with sequences whose polypeptide product is not immunologically cross-reactive (Dubbs and Bryant, 1987; Belknap and Haselkron, 1987).

Hybridization between *psb*A and *psb*D genes also occurs at very low stringency, although the gene products are antigenically distinct (Nixon et al., 1986). This hybridizable feature is usually dependent on several easy controlled factors: the probe should be a purified gene from an internal DNA fragment that is conserved in a functional and structural sense (Bryant and Tandeau de Marsac, 1988); stringent washes of blots should be avoided; low-ionic-strength washes at the hybridization temperature should not be performed. Under such conditions, even 45–55% nucleotide sequence similarities were sufficient for isolating genes (Dubbs and Bryant, 1987; Lemaux and Grossman, 1985; Murphy et al., 1987).

Synthetic Oligonucleotides

Cloning genes by utilization of synthetic oligonucleotide probes has become an increasingly popular approach in molecular biology. The advanced technology of DNA synthesis of fragments up to 100–200 base pairs in length allows screening of DNA libraries for a specific sequence even when only a small fragment of amino acid sequence of a protein product was determined.

However, due to the degeneracy of the genetic code, only in extremely rare instances will such experiments yield the desired DNA sequence. Three strategies can be employed to overcome this problem: In the first, if only a few potential sequences exist, all corresponding sequences can be synthesized and examined individually as hybridization probes. This strategy enabled the cloning of the *cpc*A gene of *Synechococcus* sp. PCC 7002 (De Lorimier et al., 1984) and the *cpc*B gene of *Cyanophora paradoxa* (Bryant et al., 1985). In the second, all potential oligonucleotide sequences are synthesized as a mixture. This mixture is radiolabeled and used for probing. The conditions for such hybridization should be determined empirically to allow only the putative perfect match to hybridize and to reduce strong competition by closely related sequences that might interfere or prevent detection of the desired signal or generate false positive signals. This strategy was used to clone genes such as the *cpc*B gene of *Synechococcus* sp. PCC 7002 (Pilot and Fox, 1984), the *pet*F gene encoding ferredoxin in *Anabaena* sp. PCC 7120 (Alam et al., 1986) and *Synechococcus* sp. PCC 7942 (Reith et al., 1986), and the *cpe*A gene encoding the α subunit of phycoerythrin in *Calothrix* sp. PCC 7601 (Mazel et al., 1986). By using two independent oligonucleotide mixtures, it is possible to minimize false-positive signals normally obtained in such experiments. Success in such experiment requires careful planning of the cloning procedure, fine tuning of the hybridization conditions, empirical determination of stringency to allow hybridization of only a perfect match sequence, and use of independent probe mixtures for a particular sequence. The third strategy utilizes unique-sequence oligonucleotide probes 30–50 base pairs in length. Codon usage data are used to predict the most likely base to be at a degenerate position, and deoxyinosine may be inserted at ambiguous codon positions (Takahashi et al., 1985). By careful selection of target sequences, the probes can be synthesized with approximately 75–80% resemblance, and hybridization may result in positive signals under restrictive conditions, as with heterologous probes. This strategy was successfully used to clone the *gvp*A gene of *Calothrix* sp. PCC 7601 (Tandeau de Marsac et al., 1985) and the *psb*E and *psb*F genes of *Synechocystis* sp. PCC 6803 (Pakrasi et al., 1988) and *Cyanophora paradoxa* (Cantrell and Bryant, 1987a).

This third method has now gained a strong momentum due to the development of the polymerase chain reaction technique (PCR), which simplifies a technically tedious protocol and makes it possible to isolate even rare sequences present at very low frequency within the largest genomes of organisms. This technique dimin-

ished concern for the region of a known polypeptide to be used to minimize the number of different oligonucleotide sequences in the synthesized mixture (due to the ambiguity of codon usage). The 5′-regions of the oligonucleotides can be designed to contain restriction sites for an easy cloning of the PCR product into a vector.

Immunological Screening

This method was developed for immunological screening of expression libraries containing the genes of interest from various sources. Antiserum is applied to the expressed gene product derived from the library and may signal the desired clone by immunological reaction followed by radiolabeled or colorimetric visualization. Since many cyanobacterial genes may be weakly expressed or not expressed at all from their own promoters in *Escherichia coli*, the screening may be facilitated by using expression vectors such as pUC plasmids (Vieira and Messing, 1982) or lambda phages (Young and Davies, 1983). This immunological approach was employed successfully in the isolation of clones encoding the *cpc*B gene of *Cyanophora paradoxa* (Lemaux and Grossman, 1983), the linker phycobiliprotein of *Nostoc* sp. PCC 8009 (Zilinskas and Howell, 1987), and the 35-kDa extrinsic protein of the oxygen evolution complex (OEC) of PS-II from *Synechocystis* sp. PCC 6803 (Philbrick and Zilinskas, 1988). In the latter case, antiserum raised against the spinach 33-kDa extrinsic protein of OEC cross-reacted with cyanobacterial the polypeptide.

This technique demonstrates the structural similarity between components of the photosynthetic apparatus of cyanobacteria and higher plants. The alpha, beta, and gamma subunits of the ATPase synthase are closely related to their plant homologs, as indicated by immunodecoration (Hicks et al., 1986). This similarity was confirmed by nucleotide sequence analysis of the corresponding genes from *Synechococcus* sp. PCC 6301 (Cozens and Walker, 1987) and *Anabaena* sp. PCC 7120 (Curtis, 1987). Similar results were obtained for components of both photosystems, the plastoquinol-plastocyanin reductase, and other enzymatic and soluble electron-transfer proteins (Vermaas et al., 1986; Nechushtai et al., 1983; Van der Vies et al., 1986).

RNA-DNA Hybridizations

This approach can use as probes only conserved genes and stable RNA molecules. Ribosomal RNAs (rRNA) are stable molecules and are widely used for determination of evolutionary development or relationships between various organisms. Chloroplast rRNA probes were suc-

cessfully used to clone the rRNA genes (*rrn*) of *Synechococcus* sp. PCC 6301 (*Anacystis nidulans*) (Tomioka et al., 1981; Tomioka and Sugiura, 1983, 1984; Kumano et al., 1983; Douglas and Doolittle, 1984a, 1984b). In this study it was discovered that the tRNA and rRNA primary and secondary structures of *Synechococcus* sp. PCC 6301 bear closer resemblance to those of chloroplast RNAs than to their counterparts in *Escherichia coli*. These similarities strengthen the endosymbiotic hypothesis, implying that eukaryotic chloroplasts are derived from an ancestral photosynthetic prokaryote (Gray and Doolittle, 1982). In addition, 14 out of 15 nucleotides at the 3′-end of the 16S rRNA are identical in *Escherichia coli*, *Synechococcus* sp. PCC 6301, and tobacco chloroplasts. Hence, it is very likely that transcripts in these three phylogenetic variable organisms have the same ribosome binding sites (Tandeau de Marsac and Houmard, 1987).

Regulation of Gene Expression

Promoter Sequences, Codon Usage, and Ribosome Binding Sites

From the cyanobacterial promoter regions elucidated so far, some homology between Pribnow boxes in *E. coli* and cyanobacteria was observed. This suggests common characteristics of RNA polymerase binding sites in both organisms. This assumption is supported by the fact that several prokaryotic genes such as *cat, lac*Z, *npt,* and *lux* are well expressed in cyanobacteria. The major variation in the Pribnow consensus is at the −35 region (Reith et al., 1986). This may be partly explained by the fact that regulation of the expression of some cyanobacterial genes involves complex processes and mechanisms like chromatic adaptation and heterocyst differentiation that may be under developmental and/or environmental regulation.

Codon usage in cyanobacteria can be evaluated by comparing the nucleotide sequence to that of the amino acid product (Shinozaki et al., 1983). Such a survey showed that codon usage is variable between unicellular and filamentous strains; correlation between the GC content in cyanobacterial DNA and the utilization of GC-enriched codons is maintained, particularly in *Synechococcus* PCC 6301; on a quantitative basis, the codon usage in cyanobacteria and *E. coli* is compatible, with the exceptions of the codons for leucine and proline.

From comparisons between the ribosome binding region in *E. coli* and the sequence of the 16S RNA of *Synechococcus* PCC 6301, some complementary sequences were identified. Also,

the binding and activity of cyanobacterial ribosomes are affected by specific inhibitors of prokaryotic ribosomal functioning. Still, in other instances, the eukaryotic-like initiation via scanning of cyanobacterial ribosomal functioning is also encountered. Thus, the mechanism used for the initiation of translation in cyanobacteria still remains an open question.

Regulation of Cyanobacterial Gene Expression

Promoter sequences and DNA binding factors have been looked at in order to understand gene regulation in cyanobacteria. Most of the genes under study belong to the major complexes related to light harvesting, photosynthesis, and nitrogen fixation, but genes involved with ATP formation and cell differentiation have also been investigated. Haselkorn and coworkers cloned the genes for a putative sigma factor and for the gamma subunit of RNA polymerase from vegetative cells of *Anabaena* PCC 7120. Both genes possess homology to their *E. coli* counterparts. Study of their inactivation may provide insights into the regulation of transcription in this cyanobacterium. The RNA polymerase of *Anabaena* PCC 7120 contains five different subunits: an α subunit that is related to an *E. coli* counterpart; a β subunit that contains the nucleotide binding site and is strongly related to the *E. coli* subunit; a β′ subunit that is weakly related to the β′ subunit of *E. coli*; a 66-kDa polypeptide that is related to the β′ subunit of *E. coli*; and a 52-kDa polypeptide that is related to another *E. coli* subunit. More than 10 cyanobacterial species were tested and found to contain a unique subunit of RNA polymerase that is not found in all other eubacteria; this places them, as a group, closer to archaebacteria (Haselkorn et al., 1983).

As mentioned in "Chromatic Adaptation," the synthesis of phycobiliproteins is regulated by light quality. Similarly, the synthesis of some of the linker polypeptides that are noncolored proteins required for assembly of phycobilisomes, depends on the light spectrum. Color mutants induced by UV mutagenesis in *Synechocystis* PCC 6701 were analyzed by Anderson and coworkers (1984). Characterization of rod and core assembly intermediates accumulating in such mutants enabled specific models to be proposed for both the assembly pathway and the final structure of the phycobilisomes in this cyanobacterium. From the various analyses performed so far with *Fremyella*, *Cyanophora*, *Agmenellum*, and *Synechocystis*, it is clear that sets of genes encoding phycobiliproteins that are polycistronically transcribed exist in cyanobacteria. The response of the various genes to alterations in light quality represents another regulatory mechanism. However, the DNA sequences involved in turning on/off the transcription of a certain gene or the binding of secondary affectors like DNA-binding proteins that inhibit/enhance expression, are still unknown.

Nitrogen fixation has been studied extensively in *Anabaena* PCC 7120 (Haselkorn, 1986). The arrangement of the genes encoding the protein components of the nitrogenase complex in *Anabaena* is different from that found in *Klebsiella*, *Azotobacter*, and *Rhodopseudomonas*. In the latter bacteria, *nif* H, *nif* D, and *nif* K encoding dinitrogenase reductase and dinitrogenase α and β subunits, respectively, are contiguous and cotranscribed from a promoter next to *nif* H. The same gene organization is observed in *Anabaena* vegetative cell DNA, except that there is just over 11 kb of DNA between *nif* D and *nif* K. Most of this DNA is not transcribed either in cells growing on ammonia or induced for nitrogenase formation. Golden et al. (1985a, 1985b) have elucidated a unique process of DNA excision during heterocyst differentiation in *Anabaena*. An 11-kb DNA fragment between *nif* K and *nif* D that contains the *xis*A gene is excised, followed by recombination between directly repeated, identical 11-bp sequences located within the coding regions of both genes. A consequence of the excision is that the ORF of *nif* D is fused to the 5′-flanking sequence of *nif* K, thus replacing 26 amino acids by 43 different amino acids. The polycistronic transcript produced by the new operon is 5 kb long and contains sequences for all three *nif* genes.

Another rearrangement occurring during *Anabaena* heterocyst formation involves the *nif* S gene, which is required for nitrogenase maturation. A DNA region adjacent to *nif* S gene (located 3′ to *nif* H in *Anabaena* vegetative-cell DNA) is rearranged via a site-specific recombination. Interestingly, the sequences at the junctions involved in the recombination differ from the sequences at the recombination junctions of *nif* D. It is suggested that two different recombination enzymes are involved in the two rearrangements.

A number of cloned genes that are turned on or off during heterocyst differentiation in *Anabaena* were used to demonstrate that the corresponding protein levels are regulated at the transcriptional level (Haselkorn et al., 1983; Wealand et al., 1989).

Machray et al. (1988) discovered an entire transposable element, IS2, near the *rbc* genes of the heterocystous cyanobacterium *Chloroglo-eopsis fritschii* CCAP1411/1b. This finding provides evidence of genetic transfer between the Gram-negative *E. coli* and cyanobacteria and may have significance in nucleotide sequence

rearrangements known to occur adjacent to the *rbc* and *nif* genes in some nitrogen-fixing cyanobacteria.

Gene Families

Gene inactivation is a useful tool for assessing the function of individual genes of a multigene family. This method was used to engineer a variety of cyanobacterial strains in which the functionality of members of gene families was analyzed. *Synechococcus* sp. PCC 7942 was examined for its three *psb*A genes by constructing mutants in which one or two out of the three genes were inactivated by an antibiotic-resistance cassette (Golden et al., 1986). This demonstrated that all three *psb*A genes are functional and that each is capable of producing sufficient Q_B protein to support the function of PS-II and photoautotrophic growth.

Golden et al. (1986) reported that out of the three members of this gene family, two functional forms of the Q_B protein are detected. Form I, produced by *psb*AI, differs by 25 residues from form II originating from *psb*AII and *psb*AIII, mostly in the amino terminus of the protein. By constructing translational gene fusions between the individual *psb*A genes and a *lac*Z gene from *Escherichia coli*, the expression of each of the *psb*A members could be followed in vivo under various illumination conditions. This experiment indicated a differential expression of the *psb*A gene family dependent on the light availability. Expression of *psb*AI is 500-fold and 50-fold greater than expression of *psb*AII and *psb*AIII, respectively, under similar illumination. If light intensity was decreased there was increased expression of the *psb*AI reporter and decreased expression of the other two genes. These results are supported by earlier reports on the relative abundance of the *psb*A transcripts in this organism when probed with antisense RNA from upstream untranslated regions of the three genes (Brusslan and Haselkorn, 1989).

Analysis of the putative promoter regions of the three *psb*A genes indicated an *E. coli*-like consensus sequence preceding the *psb*AII and *psb*AIII coding regions, whereas the *psb*AI putative promoter lacks a conserved −10 sequence (Golden et al., 1986). This difference is strengthened by the finding that the *psb*AI-*lac*Z fusion that is highly expressed in the cyanobacterium failed to produce the blue colony phenotype in *E. coli* plated in presence of the β-galactosidase indicator 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal).

These observations indicate that some cyanobacterial promoters are regulated differently than *E. coli*-like promoters and that they can be enhanced by environmental factors like high light intensity. The mechanism involved in such regulation is still unknown.

The function of two *psb*D genes coding for the D2 protein of PS-II was similarly analyzed. Since *psb*DI and *psb*C genes overlap, an additional *psb*C gene was inserted into the *psb*DII locus, thus creating a strain in which *psb*DI alone could be inactivated. This experiment demonstrated that, although not essential for viability, expression from the *psb*DI locus is required for optimal growth (Golden et al., 1989) while *psb*DII is dispensable under laboratory growth conditions.

Expression of Cyanobacterial Genes in *Escherichia coli*

A number of cyanobacterial genes were cloned, and in some instances expressed, in *E. coli* (Gurevitz et al., 1985; Gatenby et al., 1985). In most of these experiments, the genes were placed under an *E. coli* promoter to ensure binding of RNA polymerase. However, the phosphatase gene from the nitrogen-fixing cyanobacterium *Nostoc commune* UTEX 584 was cloned and was most probably expressed from its own promoter in *E. coli* (Xie et al., 1989).

Chromatic Adaptation

The response of photosynthetic organisms to light has been a major issue in photosynthesis research. The correlation between the ability to sense and capture light to many outcoming processes, including the onset of gene expression, biochemical and metabolic pathways, and the synthesis and assembly of building blocks for specific subcellular structures, was studied extensively. Cyanobacteria have developed a unique mechanism for optimizing their light-harvesting potential when exposed to changes in the spectral quality of light. This is achieved by adjusting their photosynthetic pigment system via an increase or decrease of the constituents of the photosynthetic apparatus and an adaptive rearrangement of various components involved in light capture and its transformation into biochemical energy (Post et al., 1989). Variations in the ambient light regime are reflected in various adaptation features existing in many eukaryotic algae and prokaryotic photosynthesizers. Yet, cyanobacteria are unique in that they possess phycobilisomes with which they are able to adjust their light-harvesting potential by changing the molar ratio of the phycobilins, the pigmented proteins within the phycobilisomes. This process was termed "complementary chromatic adaptation" by Bennet and Bogorad (1973) and Tandeau de Marsac (1977). It involves a complete turnover of the phycobilisome structure,

and its genetic control is currently under extensive study by cyanobacterial molecular biologists. An understanding of phycobilin synthesis will allow the recognition of genes involved in a regulatory mechanism representative of those involved in adaptation to environmental variations.

The cyanobacterium *Calothrix* sp. PCC 7601 (*Fremyella diplosiphon*) was the focus for the studies related to the chromatic adaptation. Conley et al. (1986) elucidated the genes for the synthesis of the phycobiliproteins. Three sets of the alpha and beta allophycocyanin (APC) genes, plus two sets of the alpha and beta phycocyanin (PC) genes, are all transcribed off the same strand of a 13-kb cluster on the cyanobacterial genome. The order of their arrangement seems to be conserved, as was previously found in the cyanelle DNA of the eukaryotic alga *Cyanophora paradoxa* (Lemaux et al., 1983, 1985). Pilot and Fox (1984) isolated the phycobiliprotein genes from *Agmenellum quadruplicatum* by using oligodeoxynucleotide probes based on the amino acid sequence of PC of *Agmenellum*. They succeeded in isolating the gene cluster of these proteins, determined their order to be PCβ, PCα, and found that these genes are cotranscribed in white light from an *E. coli*-like promoter. Similar results were obtained by (De Lorimier et al. (1984), who used a replicating plasmid to introduce the cloned genes APCα and β from *Cyanophora* into *Agmenellum*. The phycobilisomes were purified from the recombinant organism (De Lorimier et al., 1987), and isoelectric focusing of the phycobiliproteins permitted discrimination between *Agmenellum* and *Cyanophora* APC. Some *Cyanophora* APC could be detected in assembled bilisomes, indicating that bilin attachment to apoprotein and correct assembly into bilisome cores is possible in a heterologous host. Such an experiment paves the way for an in vitro mutagenesis program for the study of assembly and structure/function relationships of these proteins.

Differentiation

Formation of heterocysts in some filamentous cyanobacterial strains provides an excellent system for studying cell differentiation. The heterocysts are physiologically, biochemically, and structurally distinct from the vegetative cells. They can express genes that are inactive in the vegetative cells, and synthesize constituents for new metabolic pathways functioning in the assimilation of molecular nitrogen. Concomitantly, these specialized cells lose their photosynthetic capacity (structures and function) and become the nitrogen suppliers of the filament. It

should be extremely interesting to probe with a molecular marker the early detection of genes which turn on before any obvious changes are observed.

For genetic analysis of filamentous cyanobacteria, Schmetterer et al. (1986) constructed mobilizable plasmid vectors able to express the luciferase gene in *Anabaena* species. These *lux* genes may be utilized as promoter probes for studies of cell differentiation in the filaments. Indeed, this approach was recently rewarded when Holland et al. (1989) followed the induction of the *het*A gene in the premature heterocysts of *Anabaena* PCC 7120. Wolk and Elhai (personal communication) developed a remarkable technology in which transcriptional fusions put the expression of luciferase under the control of *het*A transcriptional signals. Light emitted by individual cells within filaments could be monitored by an image processing system attached to a conventional microscope. In this experiment, 9 h after nitrogen deprivation, cells emitting light were observed at intervals along the cyanobacterial filament. Only later, these cells became structurally distinguishable from the rest of the vegetative cells. It was concluded that *het*A is a gene that is expressed early in the differentiating filament of *Anabaena*. This approach provides a breakthrough in the understanding of the biochemical and genetic control of heterocysts differentiation in nitrogen-fixing cyanobacteria and may be used in the characterization of developmentally impaired mutants (Wolk et al., 1988).

Metabolism

Photosynthetic Genes

Cyanobacteria appear to be ideal organisms for the genetic study of photosynthesis since they perform photosynthesis similar to that of higher plants (Ho and Krogmann, 1982), and some species are available for genetic manipulations (Williams, 1988). However, most of the cyanobacterial strains studied are obligate autotrophs, and therefore most mutations in genes involved in photosynthesis are lethal. This problem may be overcome in two ways: 1) by the isolation of conditional lethal mutations; or 2) by the use of a facultatively heterotrophic strains which are capable of growing on an exogenous source of organic carbon (Rippka, 1972). Pakrasi et al. (1988) developed a genetic technique for molecular analysis of electron transport in PS-II in *Synechocystis* PCC 6803. Their methodology involves deletion of specific genes from the cyanobacterial genome and replacement with mutant genes prepared by site-directed

mutagenesis. In this way it is possible to investigate the role of specific amino acid residues of a given polypeptide involved in binding pigments or the role of various cofactors in the overall activity of this complex. Two important characteristics of *Synechocystis* strain PCC 6803 permit such studies: 1) their naturally occurring genetic transformation system (Grigorieva and Shestakov, 1982); and 2) their ability to grow photoheterotrophically on glucose, which is necessary for the propagation of PS-II mutants that are incapable of photosynthesis (Rippka, 1972; Jansson et al., 1987). This strain was recently used in an elegant molecular experiment aimed at the elucidation of the identity of Z, the primary electron donor to the PS-II reaction center, and D, an oxidizable PS-II component structurally resembling Z. For many years, Z and D were assumed to be plastoquinols based on a variety of physical measurements performed on the oxidized donors. However, since tyrosine radicals may yield similar physical signals to those seen in PS-II, the strongly conserved tyrosine 160 in the D2 polypeptide adjacent to the reactive histidine residue associated with P680-binding was changed to a phenylalanine (Debus et al., 1988; reference is not an exact matchVermaas et al., 1988) by site-directed mutagenesis. This change resulted in the disappearance of the electron paramagnetic response signal and led to the conclusion that tyrosine 160 in the D2 protein is the electron donor D and, by analogy, tyrosine 161 in the D1 polypeptide is electron donor Z. This discovery was achieved by site-directed modification in a cloned *psb*D gene, followed by its introduction back into the cyanobacterial genome of *Synechocystis* PCC 6803. In a different study, Pakrasi et al. (1988) examined the role of the *psb*E, *psb*F, *psb*I, and *psb*J gene products in PS-II of *Synechocystis* PCC 6803. They used site-directed mutagenesis of cloned ORFs of the *psb*EFIJ operon and demonstrated a complete loss of PS-II assembly and activity when the entire operon was deleted.

Sugar Metabolism

The primary pathway for the catabolism of endogenous glucose in cyanobacteria is the oxidative pentose phosphate cycle. This pathway is important for maintaining ATP and reducing power levels during darkness. In addition, in nitrogen-fixing cyanobacteria, this cycle may contribute reducing power for nitrogenase activity. The levels of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are sevenfold higher in heterocysts than in vegetative cells, suggesting that there is a genetic control of these enzymes in cyanobacteria which is linked to environmental effects on sugar metabolism. During the transition of a *Synechococcus* PCC 7942 culture from exponential growth to stationary phase, an increase in the specific activity of the enzyme 6-phosphogluconate dehydrogenase (6PGD) is observed. By fusing the *gnd* gene with a promoterless *lacZ* gene and transforming a *gnd⁻ Synechococcus* mutant, the growth-phase-dependent induction of 6PGD synthesis was found to be regulated at the transcriptional level. Although cyanobacteria are known to assimilate inorganic carbon via the C-3 reductive photosynthetic pathway, they also fix large amounts of carbon in the light as C-4 acids. Malate and aspartate are the major products of phosphoenolpyruvate carboxylase (PEPCase) activity. It is possible that PEPCase activity replenishes the TCA cycle with intermediates that are used for biosynthetic purposes. A genetic approach to determine the role of the enzyme in cyanobacteria may confirm this assumption. Such an experiment should involve the isolation of the *ppc* gene, followed by insertional inactivation that will indicate whether this gene is dispensable.

RUBISCO The study of rubisco structure and function was for many years an attractive challenge due to its central role in photosynthesis and CO_2 fixation. Some of the progress gained recently has been achieved by a molecular genetic approach that involves *rbc* genes derived from cyanobacteria. Since the large subunit of the enzyme is coded in higher plants by the chloroplast genome, and chloroplasts are still untransformable, it is impossible to genetically modify the large subunit of the enzyme (*rbc*L) in higher plants. Attempts to express the holoenzyme in vitro by introducing the *rbc*L and the *rbc*S genes in various ways into *E. coli* were unsuccessful. The *rbc* gene from the photosynthetic anaerobe *Rhodospirillum rubrum* has been expressed in *E. coli* and various mutations have been introduced, but the *R. rubrum* enzyme is quite different from the enzyme of higher plants. On the other hand, cyanobacteria possess a rubisco enzyme very homologous to that of higher plants, which has the substantial advantage of carrying both *rbc* genes on a single operon. This feature permitted the successful expression of rubisco from a variety of cyanobacteria in *E. coli* (Gatenby et al., 1985; Gurevitz et al., 1985; Tabita, 1988). Although many technical difficulties still limit the development of a reliable selection system to study structure/function relationships of rubisco, it may perhaps be easier to approach in transformable cyanobacteria. Meanwhile, polypeptidic chaperonins (factors assumed to play a role in the assembly of rubisco) were proposed and their involvement demonstrated by using expression vectors con-

taining *rbc* genes from *Synechococcus* PCC 6301 (Goloubinoff et al., 1989).

C_I P_UMP In cyanobacteria, the dependence of photosynthesis on external inorganic carbon is regulated by the presence of an inducible active inorganic carbon (Ci) transport system. This system operates to elevate the intracellular concentration of CO_2 so that the relatively low-affinity cyanobacterial rubisco may function at an efficiency close to that occurring at CO_2 saturation levels. Four key operational elements have been defined for this concentrating mechanism: 1) a transport system; 2) a means to energize the transport by photosynthesis; 3) a leak barrier to reduce the back flux of CO_2; and 4) a mechanism to rapidly interconvert inorganic carbon species within the cell (Reinhold et al., 1987). Interconversion of inorganic carbon species is necessary so that CO_2, the substrate required by rubisco, is formed from HCO_3^-, the carbon species that is delivered to the cell. Recently, a molecular approach to resolve the mechanism of this interconversion was initiated by several groups trying to resolve the various constituents of the concentrating mechanism. The strategy employed is the creation of mutants requiring high levels of CO_2 and using these mutants in genetic complementation experiments aimed at identifying components and genes of the system. Price and Badger (1989) succeeded in expressing a carbonic anhydrase derived from human tissues in the cytosol of *Synechococcus* PCC 7942 and created a high-CO_2 requirer. This was probably due to the conversion of cytosolic HCO_3^- into CO_2, which leaked out from the cell and prevented the accumulation of HCO_3^- within the carboxysomes. This loss of the ability to accumulate internal Ci confirmed the previous assumption that the carbonic anhydrase enzyme resided only within the carboxysome. Ogawa et al. (1987) isolated a high-CO_2 requirer (RK1) from *Synechococcus* PCC 7942, pinpointing a 42-kDa protein not synthesized in the mutant and obtained such a mutant (RKb) with *Synechocystis* PCC 6803 (J. Pierce, personal communication). Kaplan and co-workers isolated the E1 and 0221 mutants of *Synechococcus* PCC 7942, which require elevated CO_2 levels for growth. Recently, they oriented DNA sequences relative to the *rbc* locus, which seem to play a role in carboxysome formation, thus indirectly creating a requirement for higher CO_2 concentration (Friedberg et al., 1989).

Stress

Adaptation of cyanobacteria to stress conditions has been documented in several instances. *Microcystis firma*, like most other cyanobacteria, is unable to adapt to higher salt concentrations in the dark but in response to salt stress in the light, an osmoregulant, glucosylglycerol, found thus far only in cyanobacteria, is synthesized from glycogen. When the glycogen pool is depleted in the dark, glucosylglycerol is not produced in sufficient amounts. In the light, the salt-dependent accumulation of glucosylglycerol is characterized by negligible turnover in salt-adapted cells and by small but continuous leakage of this substance into the medium (Hagemann et al., 1987).

Iron is an essential component of photosynthetic cytochromes and of nonheme iron-sulfur proteins. In photosynthetic organisms, ferredoxin functions primarily as the terminal electron acceptor of the photosynthetic electron transport chain. Under conditions of moderate iron limitation, the Fe-S protein ferredoxin is replaced by the flavoprotein flavodoxin. The genes encoding ferredoxin and flavodoxin proteins were cloned from *Synechococcus* PCC 7942. Whereas the gene encoding ferredoxin is constitutively transcribed, flavodoxin was found to be transcriptionally regulated by the availability of iron. The flavodoxin mRNA was observed only in a medium low in iron, and it disappeared upon addition of iron (Laudenbach et al., 1988).

Phycocyanins represent approximately 35% of the total cell protein of *Calothrix* sp. PCC 7601. Three phycocyanin operons were characterized in this cyanobacterium by Tandeau de Marsac et al. (1988). Regulation of the third operon, *cpc*3, represents a novel response to environmental stress. The expression of the *cpc*3 operon is turned on under sulfur limitation, while *cpc*1 and *cpc*2 are switched off. The protein product of this operon lacks sulfur-containing amino acids except for those at chromophore-binding sites. This adaptation allows survival of the cell under extreme growth conditions represented by sulfur limitation (Tandeau de Marsac et al., 1988).

The availability and turnover of phosphorus may play a key role in determining the development of water blooms or of economically important nitrogen-fixing communities such as those found in rice fields. The gene *iph* (for indole phosphate hydrolase) may be useful for the study of phosphate regulation in cyanobacteria and may prove useful in the study of cyanobacterial promoter function, a subject on which there are limited data (Schneider et al., 1987).

A new UV-A/B-absorbing pigment bound to a polysaccharide core which had maxima at 312 and 330 nm was found in the cosmopolitan terrestrial cyanobacterium *Nostoc commune*. The pigment is found in high amounts (up to 10% of

dry weight) in colonies grown under solar UV radiation but only in low concentrations in laboratory cultures illuminated by artificial light without UV. Synthesis of the pigment is induced by UV light, and the pigment protects *Nostoc* from UV radiation. *Nostoc* is also capable of withstanding extreme water stress (drought). Apparently, the UV pigment can participate in water storage, since it is a polysaccharide and is located outside the cell in the polysaccharide matrix (Scherer et al., 1988).

Biotechnology

There is increasing interest in the use of cyanobacteria for biotechnology for two main reasons: 1) Because of the great similarity of their photosynthetic apparatus to that of higher plants, cyanobacteria are excellent model systems for studying oxygenic photosynthesis. 2) Mass cultivation of cyanobacteria has become a promising route for the production of large quantities of natural products of biotechnological and agricultural values.

Model Systems

Resistance to herbicides of the triazine and urea types in higher plants is a chloroplastic trait. Such resistance is a single-gene trait and may be utilized in the future for genetic engineering of crop plants. However, no reliable procedure for stable transformation of chloroplasts is available. Assuming that such a procedure will become feasible, it is necessary to better understand the phenotypic expression of the triazine-resistance gene. Thus, identical and diverse mutations have been introduced into the *psb*A gene encoding the D1 protein in the cyanobacterium *Synechococcus* PCC 7942. This reaction center II polypeptide was found to be the primary site for mutations conferring triazine resistance. The power of cyanobacterial molecular genetics was employed in the introduction of a variety of mutations to enable the elucidation of the D1 architecture in the thylakoid membranes, and the construction of a *Synechococcus* PCC 7942 strain that is partially diploid for *psb*A and heterozygous for triazine resistance (Pecker et al., 1987). From this experiment it was concluded that triazine resistance is a recessive trait in cyanobacteria. On the other hand, diuron resistance in *Synechococcus* PCC 7942 was reported to be a dominant trait by Brusslan and Haselkorn (1989).

Another important facet of future uses for cyanobacterial biotechnology may be related to rubisco. This key enzyme of CO_2 fixation controls the rate of net photosynthesis. Its complex structure and assembly, and the involvement of various factors in holoenzyme synthesis make it an extremely difficult target for genetic modification in higher plants. The simpler pathway of expression of a very similar enzyme in cyanobacteria makes it accessible to genetic manipulations which may lead in the future to better understanding of the relations between its structure and its kinetic properties.

Applications

FOOD AND NATURAL PRODUCTS. The possibility that mass cultures of specific cyanobacterial strains may be suitable for human or animal feeding is not new. The filamentous nonheterocystous cyanobacterium *Spirulina* has been part of some human diets in Africa for centuries, providing a rich source of protein, vitamins (particularly B_{12}), and the essential fatty acid gamma-linoleic acid (Cohen et al., 1987). This cyanobacterium can be grown on marginal land using saline water unsuitable for conventional agriculture. The filamentous nature of the cyanobacterium permits simple harvesting by filtration through screens. To make *Spirulina* a major food or animal feed product, its cost of production should be reduced while it is grown efficiently on a large scale. However, molecular genetics of *Spirulina* has not yet developed due to the lack of systems for transformation, transfection, or conjugation.

Cyanobacteria may be utilized for water treatment functions not practical by current mechanical or chemical means, such as purification via the incorporation of heavy metal ions.

Cyanobacteria grown on a large scale could be utilized for the production of various natural products such as polysaccharides, carotenoids, antioxidants, antibiotics, and precursors of pharmaceuticals.

Insect Control

The larvicidal gene of *Bacillus sphaericus* has been cloned and introduced into *Synechococcus* PCC 7942 by an autonomously replicating vector (Tandeau de Marsac et al., 1987). The recombinant cells produced active toxin whose level of activity against *Culex* mosquito larvae was found to be the same either in *E. coli* or in the cyanobacterium. Very often spores of *B. sphaericus* settle rapidly onto bottom mud and are removed from the larval feeding area. Introduction of such a cloned toxin into cyanobacteria, which grow on the upper layers of aquatic habitats, may provide an elegant solution for mosquito control, since cyanobacteria will persist longer in such an environment and reach target insect larvae more effectively than the spores do.

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CHAPTER 2.3

The Genus *Prochlorococcus*

ANTON F. POST

General

Until the mid-1970s the general belief was that oxygenic photosynthesis among prokaryotes was limited to the cyanobacteria with their pigment complement of chlorophyll *a* and phycobiliproteins. This concept came under fire with the subsequent discoveries of the three so-called "prochlorophytes" *Prochloron didemnii* (Lewin, 1975a; Lewin and Withers, 1975b), *Prochlorothrix hollandica* (Burger-Wiersma et al., 1989) and *Prochlorococcus marinus* (Chisolm et al., 1988), which all lack phycobilisomes but possess chlorophyll *b* as a light harvesting pigment. An additional discovery of *Acaryochloris marina* (Miyashita et al., 1996), which contains chlorophyll *d* as the main pigment, increased appreciation for the plasticity of the photosynthetic pigment-complements of prokaryotes. The photosynthetic apparatus of the above-listed species and that of its cyanobacterial counterpart differed with respect to the genes encoding the light harvesting antenna proteins, the localization of these proteins, and the regulation of photosynthetic activities (Post and Bullerjahn, 1994; LaRoche et al., 1996; Bibby et al., 2003). However, phylogenetic studies unequivocally placed them within the cyanobacterial clade (Morden and Golden, 1989; Urbach et al., 1992). Moreover, later studies have shown that these different photosynthetic machineries are to a large extent different phenotypes of a common gene pool (see below). This then suggests that broad plasticity of photosynthesis is an exclusive property of the cyanobacteria, but that the name may not provide appropriate cover for this group of fascinating organisms. At present, *Prochlorococcus* is the best-characterized organism among the "atypically pigmented" cyanobacteria, and detailed information is available on natural populations, ecological physiology, photosynthesis, nutrient acquisition, genetics and genomics. *Prochlorococcus* spp. with a cell diameter of 0.6–1.0 μm is the smallest photosynthetic organism known to date. Cells are slightly ellipsoid and have typical Gram-negative bacterial structure with a cell envelope consisting of an outer membrane, cell wall, periplasmic space and a cytoplasmic membrane. Except for photosynthetic membranes, intracellular bodies are not seen. The thylakoids are slightly appressed but as in other cyanobacteria (and in contrast to algal and higher plant chloroplasts)—the grana and stroma have no particular organization. A varying number of thylakoid membranes together form a horseshoe-shaped structure in thin-section electron microscopy pictures. Type strains are *Prochlorococcus marinus* (Chisholm et al. 1992) subsp. *pastoris* subsp. nov. strain PCC 9511 (available from the Pasteur Institute; Rippka et al., 2000) and *Prochlorococcus* strain CCMP 1378 (available from the Bigelow Marine Laboratories; Garczarek et al., 1998). *Prochlorococcus* has further proven to be an excellent tool for the study of genetic diversity and niche adaptation of microbial populations in the open ocean. At the moment *Prochlorococcus* is an organism that continues to raise great interest in disciplines as varied as comparative and functional genomics, microbiology of cyanobacteria, biological oceanography, and biodiversity. Because of its global abundance, *Prochlorococcus* is also central to studies that focus on climate change, global warming, and carbon sequestration in the surface ocean.

Habitats

First hints of the existence of *Prochlorococcus* were obtained from electron micrographs (Johnson and Sieburth, 1979) and from apparently aberrant chlorophyll signatures in pigment high pressure liquid chromatography (HPLC) analyses of tropical Atlantic Ocean samples (Gieskes and Kraay, 1983; Gieskes and Kraay, 1986; Gieskes et al., 1988). These signatures were later shown to derive from the *Prochlorococcus*-specific divinyl chlorophylls *a* and *b*. *Prochlorococcus* populations were first detected when flow cytometry was employed on board oceanographic cruises, and culture isolates confirmed the flow cytometry signatures. At present this prokaryote is known as both the smallest and

most abundant photosynthetic organism in aquatic and terrestrial ecosystems. It has been estimated to contribute 10–20% of global primary productivity. The global distribution of *Prochlorococcus* is limited with much lower abundances at more temperate latitudes (<43°S and >43°N). Although the restriction of global distribution was thought to be due to a requirement for temperatures of 18°C and above, early work showed that *Prochlorococcus* still grows at 13°C (Moore et al., 1995). Populations are much reduced in waters over the continental shelves. However, the occurrence of *Prochlorococcus* has been reported for certain coastal waters (Yahel et al., 1998; Suzuki et al., 2000). Moreover, physical forcing of subtropical and tropical water bodies into deep mixing events works to reduce *Prochlorococcus* abundances (Olson et al., 1990; Lindell and Post, 1995; Durand et al., 2001), in certain cases even rendering them as insignificant populations (Lindell and Post, 1995). Three distinct types are recognized, mostly based on their pigmentation, photosynthesis and phylogenetic properties. Cells that have low chlorophyll contents, a low chlorophyll *b/a* ratio (<0.3), and a range of relatively high irradiances for growth and photosynthesis belong to the high light (HL) adapted ecotypes (Moore et al., 1998; Moore and Chisolm, 1999). Two such ecotypes (HLI and HL2) are distinguished phylogenetically (West and Scanlon, 1999). For reasons not yet understood, the HLI and HL2 ecotypes appear to be nearly mutually exclusive and they inhabit different ocean provinces (West et al., 2001; Rocap et al., 2002). The high light adapted ecotypes are found throughout the photic zone. Cells with a high chlorophyll content and a high chlorophyll *b/a* ratio (>0.3–3) belong to the low light adapted

(LL) ecotypes (Moore et al., 1998; Moore and Chisolm, 1999). Their divinyl chlorophyll *b* permits photon scavenging in the spectral range of 480–500 nm, the wavelengths which penetrate deepest below the ocean surface. It thereby allows the LL *Prochlorococcus* types to maintain actively dividing populations at depths where other photosynthetic organisms can hardly meet their energy requirements. Populations of LL ecotypes of *Prochlorococcus* are not found in the surface layers (<50 m depth). *Prochlorococcus* spp. have not been reported from freshwater or terrestrial ecosystems.

Strains

Besides the two classified type strains mentioned above, many different isolates, but few axenic, clonal cultures of *Prochlorococcus* have been obtained. Clonal cultures were obtained from isolates from many different sites (Table 1). Most *Prochlorococcus* isolates were collected and the resulting strains maintained by teams at the Department for Civil and Environmental Engineering at the Massachusetts Institute of Technology, Cambridge, Massachusetts, United States, and the Centre d'Etudes d'Océanologie et de Biologie Marine, Station Biologique in Roscoff, France. A number of strains have been deposited at the Culture Collection of Marine Phytoplankton (CCMP) at Bigelow Marine Laboratories. The axenic strain MED4 is available as strain CCMP1378 from this collection. A second axenic strain with a genotype and phenotype similar to MED4 has been deposited in the Pasteur Culture Collection as strain PCC9511 (Rippka et al., 2000). A vast array of strains have

Table 1. Three basic seawater-based media used for the isolation and cultivation of *Prochlorococcus* spp.

K/10(−Cu)[a]		PRO2[b]		PRO99[b]	
Nutrient	Final conc.	Nutrient	Final conc.	Nutrient	Final conc.
NH_4Cl	50μM	NH_4Cl	50μM	NH_4Cl	800μM
		Urea	100μM		
NaH_2PO_4	10μM	NaH_2PO_4	10μM	NaH_2PO_4	50μM
Na_2EDTA	10μM	Na_2EDTA	1.17μM	Na_2EDTA	1.17μM
Trace metals					
$FeCl_3$	1.2μM	$FeCl_3$	1.17μM	$FeCl_3$	1.17μM
$MnCl_2$	90nM	$MnCl_2$	90nM	$MnCl_2$	90nM
$ZnCl_2$	8nM	$ZnCl_2$	8nM	$ZnCl_2$	8nM
$CoCl_2$	5nM	$CoCl_2$	5nM	$CoCl_2$	5nM
Na_2MoO_4	3nM	Na_2MoO_4	3nM	Na_2MoO_4	3nM
		$NiCl_2$	10nM	$NiCl_2$	10nM
Na_2SeO_3	10nM	Na_2SeO_3	10nM	Na_2SeO_3	10nM

[a]The K/10 (−Cu) medium was used for initial *Prochlorococcus* studies, and often this medium was Tyndallized or microwaved rather than sterilized by autoclaving (Chisholm et al., 1992).
[b]The PRO2 and PRO99 media were derived from the K/10 (Cu) medium. PRO99 is best suited for growing *Prochlorococcus* to relatively high biomass levels (Moore et al., 2002).

been collected from the northern Atlantic (e.g., strains NATL1[A] and NATL2[A]), tropical Atlantic (e.g., TATL1 and TATL2), Gulf Stream (e.g., strains MIT9311, MIT9312, MIT9313 and MIT9314), Sargasso Sea (e.g., strains SS120 [CCMP 1375], MIT9301, MIT9302, MIT9303 and MIT9401), equatorial Pacific (e.g., strains MIT9211, MIT9215, MIT9321, MIT9322 and MIT9515), south Pacific (e.g., strains MIT9107, MIT9116, MIT9123, MIT9201 and MIT9202), western Pacific (strain GP2), Arabian Sea (strain AS9601), Red Sea (strain RS810), and Mediterranean Sea (strain MED4). Coastal waters yielded strain SB from Sugura Bay and strain TAK9803-2 from the Takapoto atoll. These strains form part of the culture collection at the Station Biologique in Roscoff, France (F. Partensky, personal communication). More detailed information on *Prochlorococcus* strains, their origin, phylogenetic affiliation, physiological properties (such as pigment type and nutrient acquisition) can be found in Scanlan et al. (1996), West and Scanlan (1999), Moore et al. (1998), Moore et al. (2002), West et al. (2001), and Rocap et al. (2002). Three strains (MED4 [axenic], MIT9313 and SS120) have been submitted to full genome sequence analysis (Dufresne et al., 2003; Rocap et al., 2003). These strains show small genomes (~1.6–2.3 Mb) some of which appear to condense into an even smaller size. The various *Prochlorococcus* genomes show some surprising properties, which are discussed below, including a considerable variation in G+C content, the presence of phycoerythrin encoding genes, the presence of 1–7 gene homologs encoding the light harvesting antenna polypeptides, lack of nitrate utilization genes etc. These strain differences are so striking that one is forced to conclude that *Prochlorococcus* is a group of very closely related ribotypes with distinctly different genetic and physiological properties (see below). These properties most likely testify to the highly specialized nature of each genotype in its adaptation to a neatly defined ecological niche.

Isolation and Cultivation

The isolation and cultivation of *Prochlorococcus* is still a slightly "tricky" activity although many stumbling blocks have been removed, aided by an increased understanding of this group's ecological physiology. As an organism originating from pristine, oligotrophic environments, *Prochlorococcus* growth develops most successfully in a seawater-based medium enriched with both macro and micronutrients supplied in 10- to 1000-fold lower concentrations than in media for most other cyanobacteria. Isolations of the first

isolates SARG, MED, NATL1 and NATL2 were performed in CTPC and K/10-Cu based media (Chisholm et al., 1992), but later additional media, like PRO99, proved more effective in obtaining new isolates. A working protocol for the isolation of additional *Prochlorococcus* strains involves the following steps:

1) Ensure that seawater samples are maintained at temperatures close to ambient. Avoid both cooling and heating of samples, even by only a few degrees. 2) Protect seawater samples from exposure to high sunlight by using darkened tubing and darkened collection bottles (e.g., amber Teflon bottles). 3) Use "acid-cleaned" tubing, filtration systems, and culture glassware. 4) Filter 250 ml of seawater (without applying pressure or vacuum!) by letting the sample drip through two stacked, 0.6–1.2 μm polycarbonate filters mounted on an acid-clean Nuclepore or similar filter funnel and 1-liter acid-clean flask. 5) Add nutrients aseptically to half or full medium strength, mix gently. 6) Transfer enriched seawater (about 10 ml) to sterile, glass culture tubes (polystyrene tubes can be used at sea). And finally, 7) place tubes in light and temperature conditions that closely match the environmental conditions of the original water sample.

This protocol aims at excluding the faster developing cyanobacterium *Synechococcus* as well as picoeukaryotic algae. As some *Prochlorococcus* types are known to have a cell diameter similar to that *Synechococcus*, this procedure may bias isolation in favor of the smaller *Prochlorococcus* types. Most of the cultures should be free of contaminating phytoplankton but not of heterotrophic bacteria. Additional environmental conditions thought to provide *Prochlorococcus* with a competitive edge are the use of low light intensities and the use of blue screens to simulate the deep underwater light climate.

The apparent random success of isolation and subsequent growth and unexplained loss of cultures triggered the use modifications to the medium such as sterilization by Tyndallization rather than autoclaving, omission of certain trace elements, addition of selenium salts, omission of glassware usage, etc. (Chisholm et al., 1992). Furthermore, the filtration of seawater over a 0.2-μm polycarbonate filter or over a Whatman GF/F glass fiber filter before nutrient additions and autoclaving, is recommended. These modifications were in general found to improve success rates for individual researchers but without achieving across-the-board success. At present, many of the erratically behaving cultures have settled into a more stable and predictable routine. Many cultures can now be successfully maintained in the standard ASW medium (based

on artificial seawater and added nutrients) developed by Waterbury and Willey (1988). Moreover, the use of carefully acid-washed filtration systems for seawater, the preparation of media in Teflon or polystyrene bottles, and the use of acid washed glassware (pre-autoclaved with double distilled or "milli-Q" water) greatly stabilizes the cultivation.

Prochlorococcus spp. can further grow on solid media (e.g., PRO99 or ASW medium supplemented with good quality agar purified according to the method of Waterbury). Cells grow best when included in a 0.6–0.7% sloppy top agar prepared with medium containing 10% spent medium (v/v), i.e., culture liquid first centrifuged or gently filtered to remove the cells, passed through a sterile filter setup, and finally added to lukewarm medium-agar mix just before pouring.

Pigments and Photosynthesis

Chlorophylls

Chlorophylls *a* and *b* (the main photosynthetic pigments of *Prochlorococcus*; Chisholm et al., 1988) are found also in *Prochloron* and *Prochlorothrix* but rarely in other cyanobacteria. The dominant chlorophyll *a* and *b* species are of the divinyl-chlorophyll or 8-desethyl, 8-vinyl chlorophyll type, as opposed to the monovinyl chlorophylls found in all other known photosynthetic organisms (Chisolm et al., 1992; Goericke and Repeta, 1992). In retrospect, this characteristic, borne out by the different retention times of the two chlorophyll species in HPLC analyses, was the first hint of *Prochlorococcus*' existence in subtropical and tropical waters (Gieskes and Kraay, 1986). Divinyl chlorophyll *a* is the only chlorophyll *a* type encountered in *Prochlorococcus*, and this apparently includes the special pairs of chlorophyll *a* molecules located in the reaction centers of photosystem I and II (Goericke and Repeta, 1992). Some *Prochlorococcus*, including HL strains MIT9302, MIT9312 and LL strains MIT9211 and SS120, contain some monovinyl chlorophyll *b* next to divinyl chlorophyll *b* (Moore et al., 1995; Moore and Chisholm, 1999). Other *Prochlorococcus* have low levels of a chlorophyll *c*-like pigment, presumably Mg 3, 8 divinyl pheoporphyrin a$_5$ (Goericke and Repeta, 1992). Two ecotypes of *Prochlorococcus* are known: high light (HL)- and low light (LL)-adapted types, distinguished by their divinyl chlorophyll *a* and *b* contents. The LL *Prochlorococcus* have high divinyl chlorophyll *a* and *b* contents and high chlorophyll *b/a* ratios as compared to their HL counterparts. These types largely reflect the environment in which they thrive,

with most HL types having been isolated from well-illuminated surface layers and LL types originating from deeper layers near the bottom of the photic zone. Their pigment properties are retained by culture isolates (Moore et al., 1998; Moore and Chisholm, 1999) and relate to differences in photosynthetic antenna structure and the gene complements encoding the antenna proteins (Garczarek et al., 2000; Bibby et al., 2003).

Phycobiliproteins

Despite the early recognition that *Prochlorococcus*, *Prochloron* and *Prochlorothrix* belong to the cyanobacteria (Urbach et al., 1992), it was quietly assumed that their chlorophyll *a/b* antennae and the cyanobacterial phycobilisomes are mutually exclusive. The assumption has been put to the test with the discovery of orange fluorescence—typical of phycoerythrin—in *Prochlorococcus* samples from >100 m depth in the Pacific Ocean (Hess et al., 1996). Subsequently, the presence of the *cpeA* and *cpeB* genes encoding the α and β subunits of phycoerythrin were identified in *Prochlorococcus* CCMP1375 (= strain SS120; Hess et al., 1996). The genes are phylogenetically most closely related to phycoerythrin II genes of marine *Synechococcus*, but the genes of both the α- and β-subunits of *Prochlorococcus* phycoerythrin have stretches of high sequence dissimilarity (Hess et al., 1996). Moreover, the α-subunit of *Prochlorococcus* phycoerythrin contains only one chromophore binding site as opposed to 2–3 in marine *Synechococcus* (Hess et al., 1996). Hence this phycoerythrin was proposed to constitute a novel type named "phycoerythrin III," the main chromophore of which was identified as phycourobilin rather than phycoerythrobilin (Hess et al., 1996). A putative γ-linker polypeptide was identified for phycoerythrin III, suggesting that the phycoerythrin III subunits form a coherent functional complex (Hess and Partensky, 1999a). Phycoerythrin of *Prochlorococcus* CCMP 1375 was found in the thylakoid membranes, and its cellular levels were shown not to be affected by light intensity or growth phase (Hess et al., 1999b). Phycoerythrin is capable of light harvesting and excitation energy transfer to chlorophyll *a* (Lokstein et al., 1999). So far, phycocyanin and allophycocyanin (pigments required for energy transfer from phycoerythrin to photosystems in phycobilisome-containing cyanobacteria) have not been detected in *Prochlorococcus*. The genes encoding phycoerythrin III form a gene cluster, and their organization on the chromosome is similar to that found in marine *Synechococcus* (Hess et al., 1999b). This cluster is located at a locus <110 kb away from a gene cluster encoding photosyn-

thetic function. The two main light harvesting pigment systems in marine cyanobacteria have evolved, hypothetically, from a light harvesting antenna system of an ancestral cyanobacterium in iron-limited ocean environments (Ting et al., 2002).

Carotenoids

The carotenoid composition of *Prochlorococcus* also differs from that of other cyanobacteria. Typically, zeaxanthin, α-carotene and an additional so far unidentified carotenoid are found as the major carotenoids of *Prochlorococcus* (Chisholm et al., 1988; Goericke and Repeta, 1992). These pigments are not considered to have a light harvesting function. The cellular ratios of zeaxanthin to divinyl chlorophyll levels show dramatic change with changes in light intensity or light quality (Moore et al., 1995; Bricaud, 1999). These changes are mainly due to changes in chlorophyll contents of the cells. In contrast, α-carotene levels seem to change in concert with divinyl chlorophyll *a* in *Prochlorococcus* cultures adapted to high versus low light intensities (Moore et al., 1995). Still uncertain is how much of the zeaxanthin is associated with cell surface in *Prochlorococcus*. Much of the zeaxanthin in, e.g., *Prochlorothrix hollandica* accumulates at the cell surface and the synthesis is induced under high light conditions (Engle et al., 1991).

Pigment-Protein Complexes

Both Photosystem I (PSI) and divinyl chlorophyll *a/b*-protein (Pcb) complexes of *Prochlorococcus* CCMP 1375 (strain SS120) and CCMP 1378 (strain MED4) have been isolated and characterized (Partensky et al., 1997; Garczarek et al., 1998; Van der Staay et al., 1998). The characterization of PSI showed a high degree of similarity to PSI of other cyanobacteria. The core proteins PsaA and PsaB were identified immunologically in both strains on the basis of crossreaction with heterologous antibodies (Garczarek et al., 1998). The PsaL protein was shown to have a distinct N-terminus but to be otherwise very similar to other PsaL proteins (Van der Staay et al., 1998). *psaI* and *psaL* were identified as neighboring genes and found to be potentially cotranscribed (Van der Staay et al., 1998). PSI preparations show PSI is in the trimeric form, of which each monomer contains approximately 100 divinyl chlorophyll *a* and *b* molecules per P_{700} in both the MED4 and SS120 strains (Garczarek et al., 1998). Photosystem II (PSII) has not been characterized extensively, but electron microscopy established that PSII is

found mainly in the dimeric form (Bibby et al., 2003).

The truly unique feature of the photosynthetic apparatus of *Prochlorococcus* spp. is the chlorophyll *a/b* light harvesting antennae. The antenna proteins are made up of hydrophobic polypeptides of 31–38 kDa (Partensky et al., 1997), distinctly different from the much smaller 22–27 kDa polypeptides that form the light harvesting antennae (LHCII and LHCI) of algal and higher plants. The *Prochlorococcus* antenna proteins have molecular weights similar to those of *Prochloron didemnii* and *Prochlorothrix hollandica* (the other chlorophyll *a/b*-containing prokaryotes). Indeed, antibodies raised against LHCII crossreact to a wide range of heterologous LCHII proteins but do not recognize prokaryotic chlorophyll *a/b* antennae. Conversely, an antibody raised against the 33-kDa antenna polypeptide of *Prochlorothrix* crossreacts with the 30-kDa and 35-kDa antenna polypeptides in the same species as well as with those of *Prochloron* and *Prochlorococcus* (Bullerjahn et al., 1990; Bullerjahn and Post, 1993). As was described earlier for *Prochlorothrix* (Bullerjahn and Post, 1993; Post et al., 1993), *Prochlorococcus* showed a distinct energy coupling between chlorophyll *b* and PSI (Partensky et al., 1997). The antenna proteins are encoded by the *pcb* (prokaryote chlorophyll *b*) genes (LaRoche et al., 1996). These genes—together with the *isiA* gene which encodes a CP43'-like antenna under iron stress—have evolved from an ancestor closely related to the modern *psbC* gene encoding CP43, a PSII core antenna protein that binds chlorophyll *a* (LaRoche et al., 1996). The *pcb* genes have undergone a multiplication within the various *Prochlorococcus* ecotypes. The HL strain MED4 contains only one *pcb* gene, whereas the LL strains MIT9313 and SS120 contain 3 and 8 copies, respectively (Garczarek et al., 2000; Dufresne et al., 2003; Rocap et al., 2003). All copies of the *pcb* gene are nondifferentially expressed in these LL strains, albeit at very different steady state levels (Garczarek et al., 2001; Bibby et al., 2003).

The gene product of the sole *pcb* gene of *Prochlorococcus* MED4 forms an 18-mer antenna-protein ring that surrounds PS1 (Bibby et al., 2001b). This configuration is strongly reminiscent of the organization of IsiA antenna ring induced under Fe-stress in *Synechocystis* PCC6803 (Bibby et al., 2001a). The light harvesting antenna of *Prochlorococcus* MIT9313 is more extensive, and each photosystem has evolved with its own antenna composed of distinct proteins encoded by different *pcb* copies (Bibby et al., 2003). This is similar to the antenna partitioning in *Prochlorothrix hollandica*, where

the 30-kDa and 35-kDa chlorophyll *a/b* binding proteins are found to associate with PSI and a 33-kDa apoprotein co-purifies with PSI (Post et al., 1992). As for *Prochlorococcus* (Partensky et al., 1997), there was a distinct coupling between chlorophyll *b* and PSI in *Prochlorothrix hollandica* (Post et al., 1993). The unique partitioning of the chlorophyll *a/b* antennae in these phycobilisome-deficient oxyphotobacteria was first recognized for *Prochlorothrix hollandica* (Post and Bullerjahn, 1994b). The regulation of energy distribution in the latter involves the reversible phosphorylation of the 35-kDa antenna protein associated with PSI (Post and Bullerjahn, 1994), but this has yet to be elucidated for both the HL and LL *Prochlorococcus* spp.

As no heterotrophic capacity has been described for *Prochlorococcus* so far, the representatives of this genus appear to depend on photosynthetic activity, in support of an autotrophic lifestyle. The ambient light field that *Prochlorococcus* experiences, ranges in light intensity from 2000 µmol quanta·m^{-2}·s^{-1} (full sunlight at the sea surface) to more than four orders of magnitude less at the bottom of the photic zone. At these depths (>150 m) *Prochlorococcus* still maintains viable populations which experience a narrow spectrum centering around 490 nm (see, e.g., Veldhuis et al. [1993b], Lindell and Post [1995], Vaulot et al. [1995], and Partensky et al. [1999]). The success of *Prochlorococcus* at these depths is in part explained by the presence of divinyl chlorophyll *b* (which absorbs maximally at 488 nm; Goericke and Repeta, 1992) and the high absorption efficiency of its small cells (Morel et al., 1993). In addition to these properties, *Prochlorococcus* partitions the photic zone with HL- and LL-adapted ecotypes (Moore et al., 1998; West and Scanlan, 1999; West et al., 2001). Moreover, these ecotypes display plasticity in their pigment contents as judged from cellular fluorescence levels which increase with depth (Veldhuis and Kraay, 1990; Veldhuis and Kraay, 1993a; Veldhuis et al., 1993b). Adaptation in pigment contents was also found in culture studies of *Prochlorococcus* HL and LL strains (Partensky et al., 1993; Moore et al., 1995; Moore and Chisholm, 1999). Experiments of photosynthetic activity and growth concluded that HL *Prochlorococcus* organisms have a lower light utilization efficiency than their LL counterparts at low light intensities (though photosynthesis reaches maximal rates at higher light intensities) and are less sensitive to photoinhibition (Partensky et al., 1993; Moore et al., 1995; Moore and Chisholm, 1999). Similarly, growth rates of the HL strains peak at higher light intensities than those of the LL strains (Moore et al., 1995; Moore and Chisholm, 1999).

Nutrient Metabolism and Acquisition

Nitrogen

Little is known about the general features of *Prochlorococcus* metabolism, such as cell wall composition, carbon fixation, heterotrophy, carbon metabolism, etc. However, because *Prochlorococcus* are abundant in nutrient-depleted ocean waters, the nutrient metabolism of these organisms has received some attention in recent years. The major focus has been the ability of *Prochlorococcus* to scavenge different nitrogen (N) species and its ability to respond to N-stress. All *Prochlorococcus* strains studied are capable of ammonium acquisition, and some can utilize nitrite as the sole N-source (Moore et al., 2002). None of these *Prochlorococcus* strains is capable of N$_2$-fixation or of nitrate utilization (Moore et al., 2002). Whereas a N$_2$-fixation capacity is also absent from the closely related marine *Synechococcus*, virtually all strains of this genus are known for efficient utilization of nitrate (Moore et al., 2002). Most *Prochlorococcus* can use urea, with LL strain SS120 being a notable exception (Moore et al., 2002; Dufresne et al., 2003). No evidence has yet been found that *Prochlorococcus* uses externally supplied amino acids. Ammonium is a preferred N-source and the *amt* gene, encoding an ammonium permease, is abundantly expressed independently of the N-status of the cells (Lindell et al., 2002). A urease enzyme complex has been identified and characterized after purification (Palinska et al., 2000). In addition, genes encoding an ATP-binding cassette (ABC)-type transporter for urea were identified by similarity in the *Prochlorococcus* MED4 genome (Rocap et al., 2003). Since not all *Prochlorococcus* strains can utilize externally applied urea, this suggests that urease activity has a dual function in this genus: the acquisition of urea as a natural N-source and the removal of internally generated urea. Both ammonium and urea utilization are thought to satisfy most of the N-requirement, as they are N-sources which are readily regenerated in the surface layers of their ocean niche. Nitrite utilization is rarer and apparently limited to LL *Prochlorococcus*: strain MIT9313 was shown to carry *nirA* as well as *cysG*, which encode assimilatory nitrite reductase and sirohaem synthase, respectively (Rocap et al., 2003). Surprisingly, some *Prochlorococcus* strains have genes encoding a putative ABC-type transporter for cyanate as well as the gene for cyanate lyase, which opens the possibility for utilization of externally available cyanate (Rocap et al., 2003), which so far has not been identified as a natural N-source. Thus, all known N-sources are converted into ammonium and they are

assimilated via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway, as a glutamate dehydrogenase activity is lacking. GS was partially purified and characterized (El Alaoui et al., 2003), but GS protein levels and activity showed restricted, inconsistent change with various manipulations including changes in N-nutrition, inhibitor additions, and light and dark treatment (El Alaoui, 2001).

Like other cyanobacteria, *Prochlorococcus* can adapt to utilize different N-sources and respond to N-stress. Sensing and subsequently responding to changes in N- and C-fluxes constitute a major challenge to the cyanobacterial cell. As in other cyanobacteria, all *Prochlorococcus* strains studied so far contain P_{II} (the *glnB* gene product) and NtcA, both key players in N-stress responses (Lindell et al., 2002; Palinska et al., 2002; Dufresne et al., 2003; Rocap et al., 2003). The deduced amino acid sequence of *glnB* has distinct cyanobacterial motifs (Palinska et al., 2002), and *ntcA* sequences are highly similar to those of marine *Synechococcus* (Lindell et al., 2002). However, expression patterns suggest that the function of both genes is limited in *Prochlorococcus* PCC9511.

Phosphate

Prochlorococcus requires externally supplied phosphorus (P) in addition to combined nitrogen for sustained growth. The P-requirement of *Prochlorococcus* spp. is notably lower than that reported for other cyanobacteria (Bertilsson et al., 2003; Heldal et al., 2003). Whereas all *Prochlorococcus* spp. grow on phosphate as the sole P-source, some strains can also utilize organic P-sources, presumably aided by alkaline phosphatase activities (L. R. Moore, personal communication). Surprisingly, *Prochlorococcus* carries the genes for phosphonate uptake and utilization. Phosphonates are a utilizable P-source for marine *Synechococcus* spp. (Palenik et al., 2003). The genes encoding functions of phosphate uptake and P-stress responses have been identified. They include the genes for an ABC-type transporter and P-stress genes like *pstS* (which encodes a periplasmic substrate-binding protein with a high affinity for phosphate) and *psiP* (whose gene product has not been characterized; Scanlan and West, 2002). The adaptive response to P-stress in cyanobacteria is mediated via a histidine kinase *phoB* gene. A homologue of this gene has been identified in *Prochlorococcus* strains MED4, MIT9313 and SS120 (Scanlan and West, 2002; Mary and Vaulot, 2003). However, the *phoR* gene that encodes the response regulator PhoR was identified in strain MED4 but not in strain SS120. An apparently dysfunctional *phoR* gene was found in

strain MIT9313 (Scanlan and West, 2002; Mary and Vaulot, 2003). An additional gene, *ptrA* that encodes a putative transcriptional activator induced by P-stress, was also identified in *Prochlorococcus* (Scanlan et al., 1997; Scanlan and West, 2002). The P-stress response in *Prochlorococcus* is thus a complex matter that requires intensive further study. This is further strengthened by the observation that the phosphate availability in some marine water bodies, abundantly populated with *Prochlorococcus*, is cause to P-stress responses (Fuller et al., 2005) and growth arrest (Parpais et al., 1996) of these picocyanobacteria.

Iron and Trace Metals

Little is known about the requirements of *Prochlorococcus* for iron and trace metals. Iron (Fe) is an essential nutrient, and depth profiles of Fe-concentrations in the surface ocean often resemble those of the macronutrients, with depleted levels near the surface and accumulation at depths below the photic zone (Martin and Gordon, 1988). *Prochlorococcus* populations were found to be Fe-limited in the eastern equatorial Pacific (Mann and Chisholm, 2000). An Fe-induced transcriptional activator gene (*fur*) was identified in both the MED4 and MIT9313 strains (Rocap et al., 2003). Genes that are potentially controlled by Fur include *isiB* (flavodoxin), genes encoding Fe-storage in the form of ferritin, and an ABC-type iron transporter. Many of these genes have a putative Fur box motif in the promoter region. Typically, *Prochlorococcus* lacks the genes involved in the synthesis of Fe-complexation via siderophores (Rocap et al., 2003). Both *isiB* and one of the *pcb* genes are upregulated in strains MIT9313 and SS120 under Fe-stress (Bibby et al., 2003). Trace metal studies have shown a high sensitivity of *Prochlorococcus* to copper—LL strains more so than their HL counterparts—with toxicity becoming apparent at free Cu^{+2} concentrations of <10 pM (Mann et al., 2002). This sensitivity is greater by orders of magnitude than that found for marine *Synechococcus* and eukaryotic algae, and it may be a contributing factor in the global distribution of *Prochlorococcus* (Mann et al., 2002). *Prochlorococcus* has a specific requirement for the trace metal cobalt, needed in enzymes like carbonic anhydrase, which cannot be replaced by, e.g., zinc (Saito et al., 2002). Cobalt scavenging may involve ligands secreted by the *Prochlorococcus* cells, as cobalt uptake occurs at elevated rates in aged, conditioned medium as compared to uptake rates in fresh growth medium which permits uptake of Co^{+2} only (Saito et al., 2002).

Genomics

One of the exciting recent developments in the biology of *Prochlorococcus* is as a result of the wide attention this genus has received in genome sequencing projects. This interest was generated by the recognition that *Prochlorococcus* is one of the major primary producers in the sea and thus a major contributor to drawdown of atmospheric CO_2 into particulate organic matter. Over the last decade much attention has been paid to remedy elevated levels of CO_2 (and thus global warming) by increased marine productivity supported by large-scale fertilization of the surface ocean. Genome sequencing on the one hand provides in depth understanding of the gene pool underlying the global success of the ubiquitous unicellular cyanobacteria *Prochlorococcus* and the related *Synechococcus*. Three *Prochlorococcus* genomes, those of the strains MED4, MIT9313 and SS120, have been sequenced in full (Dufresne et al., 2003; Rocap et al., 2003). Additional genome sequencing for strains MIT9312 and NATL2A has been undertaken, but these sequences may be partial with an approximately 95% coverage. Although they are among the smallest genomes known, the genomes of different *Prochlorococcus* strains vary considerably in size (Table 2). As a result, the LL strain MIT9313 carries ca. 50% more genes than the HL strain MED4 (Rocap et al., 2003). Moreover, the MIT9313 is relatively rich in G+C as compared to MED4. However, another LL strain, SS120, is characterized with a genome size and G+C content more similar to that of MED4 than to the other LL strain MIT9313 (Dufresne et al., 2003). The genome of *Prochlorococcus* strain MED4 is so far the smallest genome known for a photosynthetic organism, and it is tempting to speculate that this genome size may be close to the size of the gene pool minimum needed by a free-living photosynthetic organism (Rocap et al., 2003). However, a comparison of the (predicted)

genes within the available *Prochlorococcus* genomes shows that *Synechococcus* spp. share a common core of about 1310 genes (Dufresne et al., 2003; Rocap et al., 2003). This finding suggests that these genes carry out essential cell functions, whereas the remaining 300–1000 genes encode proteins involved in cellular interactions with the environment. These observations have further contributed to the establishment of *Prochlorococcus* as a model organism in comparative and functional genomics studies.

The small genome size of *Prochlorococcus* spp. may have resulted from active genome condensation, which started in an ancestor probably resembling modern day marine *Synechococcus*. Comparison of *Prochlorococcus* genomes relative to that of *Synechococcus* shows that rapid degeneration of genes—obvious from frameshifts and other mutations—preceded the eventual omission of these genes from the genome (Hess et al., 2001; Scanlan and West, 2002; Rocap et al., 2003). An example of such a deletion event explains why *Prochlorococcus* spp. are incapable of nitrate utilization. Figure 1 shows the genome region responsible for nitrate assimilation in *Synechococcus* WH8102. These genes encode a nitrate permease, the nitrate and nitrite reductases, and all enzymes required for the synthesis of their molybdopterin and sirohaem cofactors. A deletion event has removed the nitrate utilization genes from the *Prochlorococcus* MIT9313 genome, but the nitrite utilization genes *nirA* and *cysG* were retained. The LL strain MIT9313, phylogenetically related to marine *Synechococcus* (Moore et al., 1998), is indeed capable of utilizing nitrite but not nitrate (Moore et al., 2002). An additional deletion event occurred in MED4 and may have happened during the evolution of these more recent HL types. As a result of this event, MED4 lost the *nirA* gene and thus nitrite reductase activity, whereas the *cysG* gene was retained, as cells still require sirohaem for sulfite reductase activity (a conserved function). The HL strains are all incapable of nitrate and nitrite utilization (Moore et al., 2002). Similar scenarios were used to explain the omission of, e.g., phycoerythrin synthesis and phosphate stress genes (Hess et al., 2001; Scanlan and West, 2002; Rocap et al., 2003). These deletion events no doubt laid the foundation for the establishment of *Prochlorococcus* ecotypes with very high similarity at the 16S rDNA level but very different physiological properties finely tuned to very different ocean niches. However, gene duplication and acquisition of foreign DNA through lateral gene transfer also contributed to development of successful ecotypes. The most striking example of gene duplication is the *isiA*-related *pcb* gene in LL type *Prochlorococcus*, which is found with two copies in MIT9313 and

Table 2. Basic properties of the whole genome sequences of *Prochlorococcus* strains MED4, SS120 and MIT9313.

Prochlorococcus strain	MED4	SS120	MIT9313
Genome size (bp)	1,657,990	1,751,080	2,410,873
G+C content (%)	30.8	36.4	50.7
Transfer RNAs (no.)	37	40	43
Gene models (no.)[a]	1716	1884	2275
Genes shared (no.)[b]	1314	1308	1358

Abbreviation: bp, base pairs.

[a]Gene models are the total number of predicted genes (known, conserved hypothetical, and hypothetical genes).

[b]Genes shared are the number of genes in each genome that have an ortholog in the other *Prochlorococcus* genomes and in marine *Synechococcus* WH8102 (Dufresne et al., 2003; Palenik et al., 2003; Rocap et al., 2003).
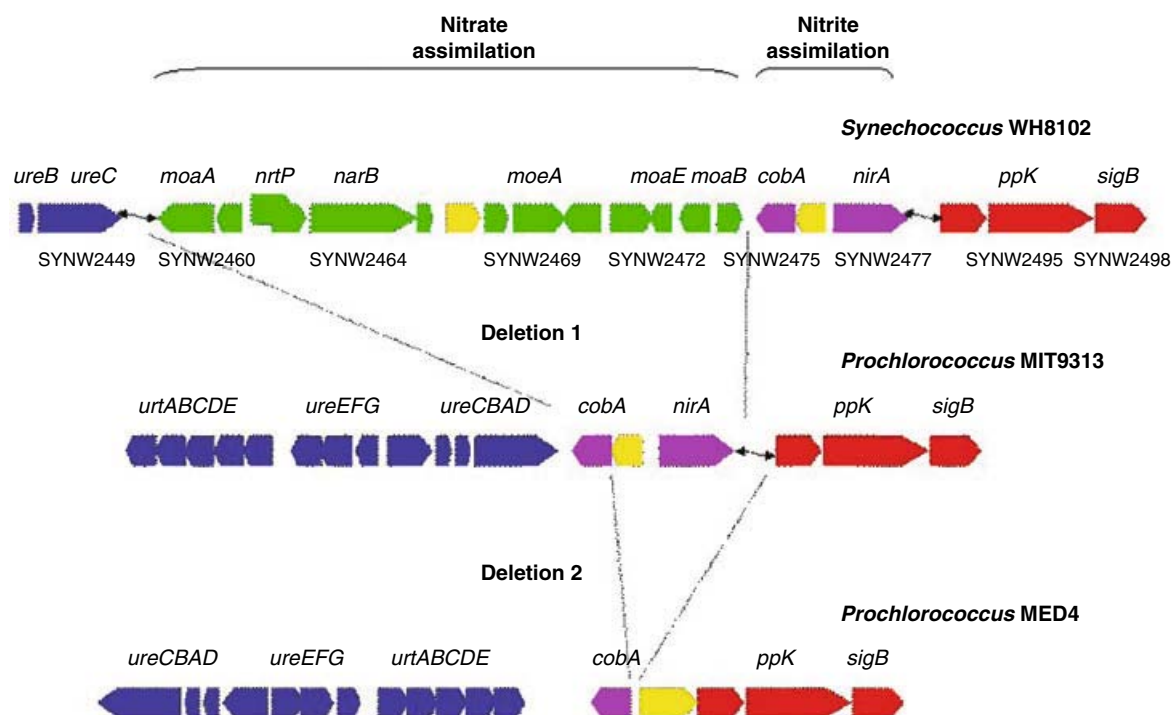
Fig. 1. A comparison of nitrate and nitrite assimilation gene arrangements in a genomic region in *Synechococcus* WH8102 which contains further N-acquisition genes, as compared to similar genomic regions in the LL strain *Prochlorococcus* MIT9313 and the HL strain *Prochlorococcus* MED4. The *ppK*/*sigB* genes and *pyrG* (not shown) were chosen as marker genes indicating the flanking regions of the genomic regions. The genes indicated by the deletion events were lost from the genome and not translocated to other regions. Coding regions of known and putative genes are as represented. Black arrows indicate the direction of transcription, green arrows indicate genes involved in the assimilation of nitrate, pink arrows indicate genes involved in nitrite assimilation, and dark-blue arrows indicate genes needed for urea utilization. Known gene names are given above and open reading frame numbers (SYNWxxx) below the black arrows.

eight copies in strain SS120 (Garczarek et al., 2000; Bibby et al., 2003; Rocap et al., 2003). The expression of multiple *pcb* genes (Garczarek et al., 2001) provides the structural basis for different light harvesting antennae associated with each photosystem (Bibby et al., 2003) and for unusually high chlorophyll *b*/*a* ratios in these *Prochlorococcus* organisms (Moore et al., 1998; Moore and Chisholm, 1999). The expression of multiple *pcb* genes is thought to explain the success of LL *Prochlorococcus* types at depths where light is <0.1% of incident light at the surface. The genomic section between *mutS* and *gyrB* genes has been well conserved among marine *Synechococcus* and *Prochlorococcus*. However, the MIT9313 genome carries an uncharacteristic insertion of 33 genes—most encoding functions in lipopolysaccharide biosynthesis—which have a very different G+C content from that of the neighboring genome sections. Acquisition of this gene cluster in lateral gene transfer has been hypothesized (Rocap et al., 2003). Similarly, the genes involved in inorganic carbon acquisition are similar to those of the gamma-proteobacterium *Acidithiobacillus fer-*

roxidans rather than to typical cyanobacterial-type genes, again suggesting a lateral transfer of foreign DNA to the *Prochlorococcus* genome (Rocap et al., 2003).

The whole genome sequences available have altered our previous view of *Prochlorococcus* as a coherent genus specialized to meet the extreme environmental conditions imposed by stratified subtropical and tropical ocean. This view has changed to one of a genus displaying an enormous versatility in G+C content, codon usage, gene pool and gene expression for individual species or ecotypes, in which very few if any "luxury" genes are present. These are thereby optimally equipped to successfully invade and dominate in virtually every niche created by the steep and inverse gradients of light and nutrients in the ocean's photic zone.

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